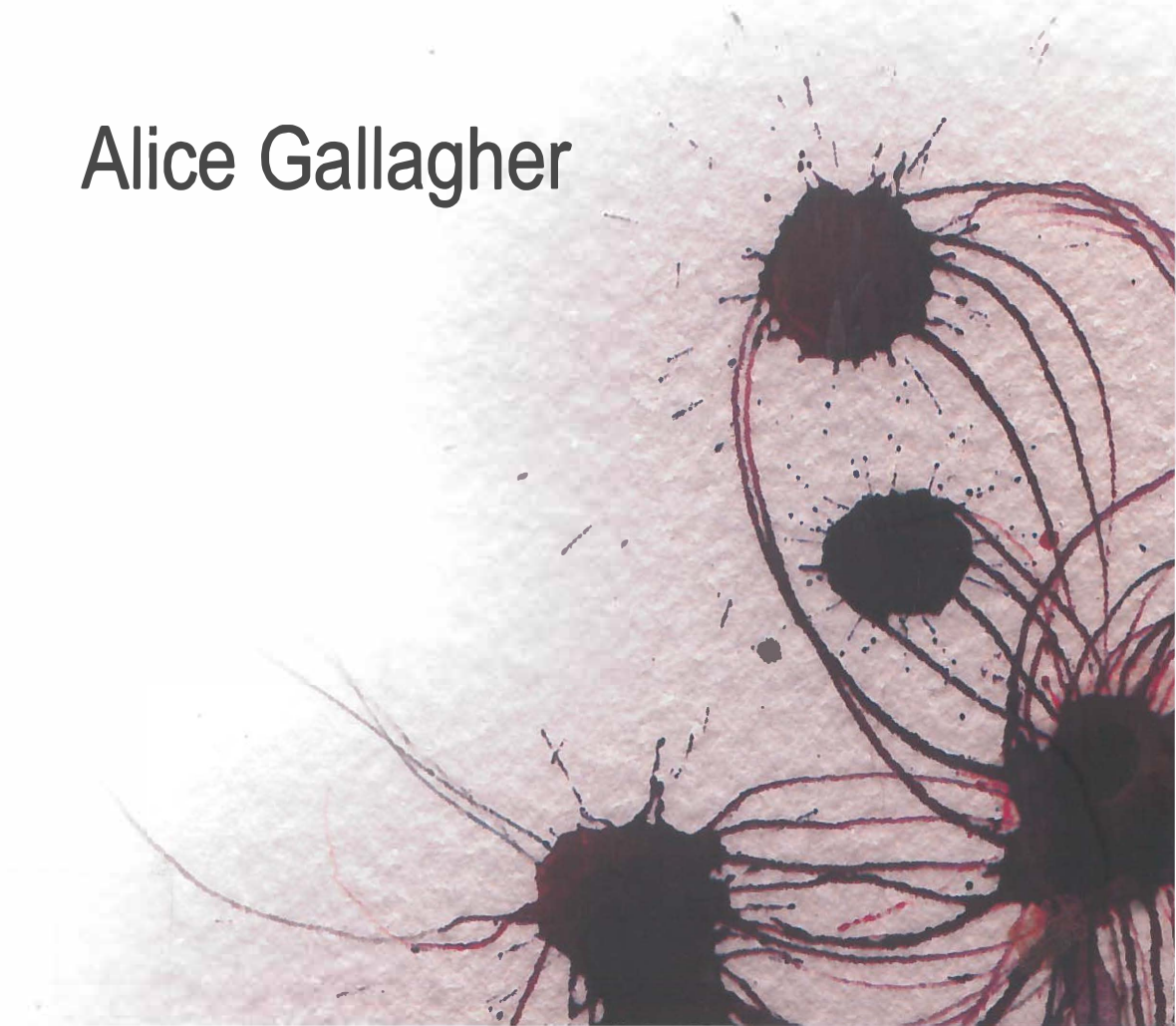
Classical Hodgkin lymphoma

Gallagher, Alice

Classical Hodgkin lymphoma:
Investigation of the disease pathogenesis with a focus on viral involvement

Alice Gallagher



Classical Hodgkin lymphoma:

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Alice Gallagher

Stellingen behorende bij het proefschrift

Classical Hodgkin lymphoma: Investigation of the disease pathogenesis with a focus on viral involvement

Alice Gallagher, 18 april 2007

1. Serum Epstein-Barr virus (EBV) genome levels show promise as a prognostic marker in EBV-associated Hodgkin lymphoma (HL).

2. EBV is an unlikely 'hit and run' candidate in EBV-negative HL.

3. EBV-negative young adult HL: An infectious aetiology cannot be excluded.

4. There is no evidence that polyomaviruses and herpesviruses are involved in EBV-negative HL.

5. The putative agent in EBV-negative HL remains elusive.

6. Serial analysis of gene expression in HL may identify novel therapeutic targets.

7. To be absolutely certain about something, one must know everything or nothing about it (Olin Miller).

8. The important thing is never to stop questioning (Albert Einstein).

ISBN: 978-90-367-2997-0

Cover: Designed by Moyra Campbell

Printing: PrintPartners Ipskamp B.V., Enschede, The Netherlands

RIJKSUNIVERSITEIT GRONINGEN

Classical Hodgkin lymphoma:
Investigation of the disease pathogenesis with a focus on viral involvement

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. F. Zwarts,
in het openbaar te verdedigen op
woensdag 18 april 2007
om 14.45 uur

door

Alice Gallagher

geboren op 30 augustus 1964
te Glasgow, Schotland

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The work described in this thesis was carried out mainly at the LRF Virus Centre, Institute of Comparative Medicine, University of Glasgow, Glasgow, UK. Work described in chapter 7 was a collaborative project undertaken with the Department of Pathology and Laboratory Medicine, University Medical Center Groningen, University of Groningen, The Netherlands.

The printing of this thesis was financially supported by Professor David Onions.

The studies described in this thesis were financially supported by a LRF Specialist Programme Grant.

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CHAPTER 1

INTRODUCTION

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1.1. Hodgkin lymphoma - an overview

Hodgkin lymphoma (HL) is a malignant disease which accounts for around 30% of all lymphomas (WHO 2001). The disease is characterised by the presence of Hodgkin and Reed-Sternberg (HRS) cells, the proposed malignant cells of the disease, in a polymorphous cellular background. The presence of these cells within an appropriate cellular background is necessary for the diagnosis of HL as Reed-Sternberg-like cells are also found in other conditions (Strum *et al.,* 1970). A striking feature of this disease is the scarcity of the malignant cells usually less than 1% within the tumour mass. The rarity of these cells has made characterisation of this disease a difficult task for researchers. Advances in molecular techniques over the last decade have enabled HL research to move forward substantially as will be discussed in subsequent sections of this thesis.

The disease was first described in 1832 by Thomas Hodgkin after a study involving the examination of seven lesions of the lymphatic glands and spleen (Hodgkin 1832). The term Hodgkin's disease (HD) was applied some 30 years later following examination of cases for clinical and gross pathological findings (Wilks 1865). The histology of HD was first described by Sternberg in 1898 and detailed further by Reed in 1902 (Sternberg 1898;Reed 1902). The authors described the large bi-lobed or multinucleated cells which are characteristic of the disease. Both also believed the disease was infectious in nature; Sternberg proposed a Mycobacterial infection and Reed some other unknown agent. In more recent years the characterisation of the HRS cells as being derived from pre-apoptotic germinal centre B-cells has led to this disease entity being named HL as opposed to HD but these terms are synonymous (Kuppers *et al.,* 1994;Kuppers and Rajewsky 1998).

Around 90% of HL patients are cured from the disease however treatment strategies lead to late complications in long term survivors (Draube *et al.,* 2006). These include secondary malignancies, loss of fertility, cardiovascular and lung

diseases (Ng and Mauch 2004). Although the cure rate is high in HL some patients still have a poor prognosis; therefore, an improvement in cure rate and reduction in long term toxicity is desirable and perhaps attainable if novel treatment strategies can be employed (Connors 2005).

1.2. Classification of HL

HL is subdivided into two distint entities know as classical HL (cHL) and Nodular Lymphocyte-Predominant HL (NLPHL) (Harris *et al.,* 1994). The World Health Organisation has classified cHL further into 4 subtypes based on the 'Revised European-American Classfication of Lymphoid neoplasms'. These are nodular sclerosis (NS), mixed cellularity (MC), lymphocyte-rich classical (LRC) and lymphocyte depleted (LD) HL. (Harris *et al.,* 1994;WHO 2001). The descriptive features of NLPHL and cHL are given below thus highlighting the distinction between these disease entities; however, the focus of this thesis is on cHL.

Nodular Lymphocyte Predominance HL

NLPHL accounts for around 5% of all HLs. Sites of presentation include the axillary, cervical or inguinal lymph nodes. Involvement of the spleen, mediastinum or bone marrow is rare (WHO 2001). This very distinctive subtype is characterised by the presence of " popcorn" or lymphocytic and/or histiocytic (L&H) cells and these neoplastic B-cells are scattered in nodular or nodular and diffuse infiltrates containing histiocytes and small lymphocytes. In the majority of cases the L&H cells are positive for CD20, CD79a, Ki-67, BCL6 and CD45 (WHO 2001). A large proportion of cases are also CD75 positive (Anagnostopoulos *et al.,* 2000). In contrast to cHL, the L&H cells are generally CD15, TARC (CCL17) and CD30 negative although CD30 positivity is sometimes present (WHO 2001). A characteristic feature is that CD3+ and CD57+ T cells surround most of the L&H cells. The transcription factor Oct 2 is expressed in L&H cells in conjunction with its co-actvator BOB.1 (Laumen *et al.,* 2000;Stein *et al.,* 2001). Oct 2 and BOB.1

may be useful in the differential diagnosis between NLPHL and cHL as tumour cells in cHL cases do not co-express these proteins. Identical immunoglobulin (Ig) gene rearragements can be detected in the L&H cells from a given case and analysis of the Ig heavy chain variable regions shows evidence of ongoing somatic hypermutation. Ig mRNA transcripts are detectable and rearrangements appear functional in the majority of L&H cells (Marafioti *et al.,* 1997;Kuppers *et al.,* 1998). The postulated cell of origin in NLPHL is the germinal centre B-cell at the centroblastic stage of differentiation. In contrast to a proportion of cHL cases, the L&H cells of NLPHL show no evidence of latent Epstein-Barr virus (EBV) infection (Anagnostopoulos *et al.,* 2000). NLPHL is a relatively indolent disease compared with cHL (Orlandi *et al.,* 1997).

Classical HL

cHL accounts for 95% of all HLs. The common sites of presentation in the lymph nodes are the cervical region followed by the mediastinal, axillary and paraaortic regions. Mediastinal involvement is frequently associated with the NS subtype whereas in the MC subtype abdominal and splenic involvement are relatively more common. In 40% of patients constitutional 'B' symptoms including tiredness, weight loss, fever and night sweats are also present (Selby and McElwain 1987).

The neoplastic tissue in all subtypes of cHL is composed of mononuclear HRS cells in a polymorphous cellular background. This background contains lymphocytes, histiocytes, plasma cells, neutrophils, fibroblasts, collagen fibres and eosinophils. The HRS cells in cHL have identical genetic and immunophenotypic features, however, the characteristics of the infiltrate, the morphology of the HRS cells and association with EBV differ between the subtypes (WHO 2001). The characteristic immunophenotype of the HRS cell is CD30+ in virtually all cases (Stein *et al.,* 1981;Stein *et al.,* 1985) and CD15+ in the majority of cases (Stein *et al.,* 1981;Stein *et al.,* 1982). CD20 can be detected in around 40% of cases but this is variable and only a minority of neoplastic cells are positive. J chain is consistently negative as is CD75 and macrophage specific markers. CD80, CD86,

CCL17 and FSCN1 (dendritic cells), MUM1 (plasma cells) and GATA-3 and T-bet (T-cells) are also detected (Re *et al.,* 2005;Atayar *et al.,* 2005). Generally CD45 is negative and the B-cell associated antigen CD79a is rarely detected on the HRS cells.

B-cell specific activator protein, which is a product of the PAX5 gene, has been detected in the majority of cases in most studies (Foss *et al.,* 1999). This B-cell commitment and maintenance transcription factor is present even though most typical B-cell genes are down regulated in HRS cells (Schwering *et al.,* 2003b). Another regulator of the B-cell specific gene expression programme, E2A, is also usually expressed by HRS cells but most of its target genes are not expressed (Hertel *et al.,* 2002;Mathas *et al.,* 2006). Detection of the transcription factor Oct2 and/or its co-activator BOB.1 is absent (Stein *et al.,* 2001). A recent study showed expression of MAL, a gene overexpressed in mediastinal (thymic) large B-cell lymphoma in a proportion of cHL cases; expression correlated with the NS subtype of the disease and poor prognosis (Hsi *et al.,* 2006). MAL mRNA was originally identified during T-cell development and encodes a proteolipid believed to participate in membrane microdomains transport machinery, stabilization and signal transduction (Copie-Bergman *et al.,* 2002).

The HRS cells of cHL overexpress a variety of cytokines and chemokines which may enable them to create their own microenvironment and subsequently increase their growth and survival (Skinnider and Mak 2002). Expression of Interleukin (IL)-2, IL-5, IL-6, IL-7, IL-9, IL10, IL-13 and IL13 receptor, transforming growth factor-β (TGF-β), lymphotaxin-α, granulocyte macrophage colony-stimulating factor, eotaxin and CCL17 can be detected (Kadin *et al.,* 1990;Samoszuk and Nansen 1990;Herbst *et al.,* 1996;Kapp *et al.,* 1999;Teruya-Feldstein *et al.,* 1999;van den Berg *et al.,* 1999;Jones *et al.,* 2000;Skinnider *et al.,* 2001). The increased expression of eotaxin may partially explain the presence of many eosinophils within some HL tumours (Teruya-Feldstein *et al.,* 1999) and the fibrosis seen may be due to TGF-β (Kadin *et al.,* 1990). A recent report suggests that IL-9 is related to the

presence of eosinophils and mast cells in HL and is important in inflammatory infiltrate formation (Glimelius *et al.,* 2006).

HRS cells also express a number of molecules which are important for T-helper (Th) cell-B cell interaction. The T-cell infiltrate which consists primarily of Th2 cells, surrounding the HRS cells may result from increased expression of CCL17 (van den Berg *et al.,* 1999). These T cells often rosette around the HRS cells and may be contributing to an environment that favours survival of the HRS cells. The recent demonstration of an abudance of regulatory T-cells in HL, which induce an immunosuppressive environment, may explain the ineffective clearance of HRS cells by the immune system (Marshall *et al.,* 2004). In addition, attraction of lymphocytic subsets to the HRS cell micro environment by chemokines may lead to differences in the distinctive subtypes of HL (Poppema and van den Berg 2000;Maggio *et al.,* 2002).

Studies of oncogenes and tumour suppressor genes in the pathogenesis of cHL have been hampered by the scarcity of the HRS cells within a tumour mass. Advances in microdissection techniques and molecular analyses have enabled a more comprehensive investigation of the HRS cells. Investigation for translocations of the proto-oncogenes bcl-2 and bcl-6 showed that they are rarely detected in HRS cells (Gravel *et al.,* 1998;Seitz *et al.,* 2001). Only few cases showed mutations of the tumour suppressor p53 (Montesinos-Rongen *et al.,* 1999).

Chromosomal gains have been demonstrated in cHL frequently involving 2p, 9p, 12q, 16p, 17q and 20q with a loss of 13q (Joos *et al.,* 2003;Chui *et al.,* 2003). The proposed target genes of 2p, 9p and 12q are *c-REL, JAK2 and MDM2* respectively (Kupper *et al.,* 2001;Joos *et al.,* 2003). The nuclear localisation of c-REL correlates with the gain of 2p and this may be contributory to constitutive NF-κB in cHL (Barth *et al.,* 2003).

1.3. Origin of the HRS cell

For many years the origin of the HRS cell was unknown. Earlier studies attempting to clarify if HRS cells were of lymphocytic origin, used both polymerase chain reaction (PCR) and Southern blot analysis to analyse DNA extracted from whole HL tumour tissue and gave inconclusive results (Gledhill *et al.,* 1990;Tamaru *et al.,* 1994;Manzanal *et al.,* 1995;Orazi *et al.,* 1995;Yatabe *et al.,* 1996;al Saati *et al.,* 1997). Micromanipulation of HRS cells from frozen tissue sections and subsequent Ig gene PCR showed that the HRS cells were clonal B-cells (Kuppers *et al.,* 1994;Kanzler *et al.,* 1996a). The number of HL cases in these studies was small although a larger study confirmed these findings (Kanzler *et al.,* 1996b). The detection of mutations rendering the Ig genes non functional and the high number of somatic mutations led to the conclusion that the HRS cells were derived from pre-apoptotic GC B-cells. HRS cells do not express B-cell receptor (BCR) which would signal cell death in normal B-cells. The HRS cells do survive the germinal centre reaction and are therefore resisting apoptosis (Marafioti *et al.,* 2000). Despite a B-cell origin downregulation of B-lineage specific gene expression has been demonstrated in HRS cells (Schwering *et al.,* 2003b). This loss of B-cell phenotype may help explain the survival of HRS cells without a BCR as discussed further in section 1.9.

Two studies also describe the rare detection of T-cell receptor gene rearrangements in cHL and conclude that the disease can infrequently exist as a T-cell lymphoma (Seitz *et al.,* 2000;Muschen *et al.,* 2000a).

1.4. Epstein-Barr virus

Biology of EBV

Epstein-Barr virus (EBV) is a human B-lymphotropic herpesvirus which is carried in a persistent state by over 90% of the world population. The virus was originally

identified in cultured lymphoblasts derived from Burkitt's lymphomas (BLs) of African patients (EPSTEIN *et al.,* 1964). Primary infection generally occurs in early childhood and is asymptomatic. Late exposure and primary infection of the virus in adolescence or young adulthood results in the onset of infectious mononucleosis (IM)/glandular fever in approximately 25% of cases (Crawford *et al.,* 2006).

EBV is a γ herpesvirus and all members of this subfamily of herpesviruses have the ability to replicate in lymphoid cells *in vitro.* There are two distinct phases of the herpesvirus lifestyle known as lytic and latent phases. In the lytic cycle, viral particles are produced and the host cell DNA protein synthesis can be shut of leading to host cell death (Wagner and Roizman 1969). The ability to establish a latent infection, in which a limited number of viral genes are expressed, is characteristic of herpesviruses.

EBV latent gene expression

In B-cells three patterns of expression of the EBV latent proteins have been described, designated Lat I, Lat II and Lat III. In Lat I only the EBV nuclear antigen (EBNA)-1 protein is expressed. In Lat II, both EBNA-1 and the latent membrane proteins (LMPs) are expressed and in Lat III all of the EBV latent gene products are present (Rowe *et al.,* 1992). Various malignancies have been associated with different patterns of EBV gene expression. Lat I expression is seen in BL (Rowe *et al.,* 1992) whereas Lat III latency is generally observed in lymphomas in immunosuppressed persons and lymphoblastoid cell lines (Young *et al.,* 1989). In undifferentiated nasopharyngeal carcinoma an intermediate pattern or Lat II is described (Young *et al.,* 1988).

Mechanisms of HRS cell survival in EBV-associated HL

EBV is detected in and localised to the HRS cells in a proportion of HL patients, which varies in relation to geographical local (Armstrong *et al.,* 1992;Jarrett *et al.,* 1996). EBV detected in HL lesions is clonal indicating infection and expansion of a single cell (Weiss *et al.,* 1987;Jarrett *et al.,* 1991;Pallesen *et al.,* 1991a). These

cases are deemed EBV-associated (Armstrong *et al.,* 1992). The latent gene products EBNA1, LMP1 and LMP2 are expressed with a lack of EBNA 2 giving a Lat II pattern of expression (Pallesen *et al.,* 1991a).

It is now widely accepted that EBV is involved in the pathogenesis of the EBV-associated cases of HL (IARC 1997). The EBV LMP-1 is expressed in these cases and LMP-1 has been shown to transform rodent fibroblasts in vitro. It is therefore likely that this major transforming protein of EBV is involved in the transformation of HRS cells. A key function of LMP-1 is it mimics an activated CD40 receptor leading to activation of the transcription factor NF-κB (Kilger *et al.,* 1998). Expression of LMP-1 in the EBV-associated cases of HL is therefore an additional method of NF-κB activation (Eliopoulos *et al.,* 2003). NF-κB is discussed further in section 1.5. The expression of LMP2a by EBV may also play a role in the rescue of HRS cells by mimicking a BCR. The survival of LMP2a transgenic B-cells has been demonstrated in the absence of a BCR (Caldwell *et al.,* 1998;Casola *et al.,* 2004). Three independent studies have also recently demonstrated that EBV has the ability to rescue BCR-deficient germinal centre B-cells from apoptosis (Mancao *et al.,* 2005;Chaganti *et al.,* 2005;Bechtel *et al.,* 2005). These studies add substantial support to the idea that EBV plays a role in rescuing HRS cells, or their precursors, from apoptosis in germinal centres.

1.5. Other mechanisms of HRS cell survival

The exact mechanism responsible for the survival of the HRS cells in cHL is still unclear however a number of factors are known to be involved in their rescue from apoptosis.

De-regulated expression of inhibitors of apoptosis (IAP) family members may confer apoptosis resistance in HL. The HRS cells of cHL co express CD95 (Apo-1/FAS) and CD95L (Metkar *et al.,* 1999;Verbeke *et al.,* 2001). Apoptosis of normal

GC B cells is in part due to CD95 signalling (Liu *et al.,* 1997); however, HRS cells appear resistant to CD95 mediated apoptosis (Re *et al.,* 2000). In a small number of cases this can be explained by the presence of CD95 gene mutations (Muschen *et al.,* 2000b). A more frequent scenario is likely to be the inhibition of CD95 signalling by cFLIP expression in HRS cells (Mathas *et al.,* 2004). HRS have also been shown to express the X-linked inhibitor of apoptosis (XIAP), which inhibits activation of caspase-3 mediated cell death (Kashkar *et al.,* 2003). Expression of cIAP1, cIAP2 (Zheng *et al.,* 2004) and survivin (Garcia *et al.,* 2003) have also been demonstrated to a variable extent in HL cell lines and primary tissue. A more recent study found that cIAP2 is expressed in the HRS cells in 20 of 23 cases of cHL cases by in situ hybridisation and that this inhibitor of caspase 3 is inducible by CD30 stimulation. The authors further suggest that cIAP2 contributes to apoptosis resistance in HRS cells and that newly developed IAP inhibitors may be beneficial for the treatment of patients with cHL (Durkop *et al.,* 2006).

NF-κB is a transcription factor that regulates the expression of many effector molecules including growth factors, cytokines and anti-apoptotic proteins in response to ligation of surface receptors. It is now widely accepted that constitutive activation of NF-κB in the HRS cells plays a critical role in escape from apoptosis. Both cFLIP and XIAP are NF-κB target genes and therefore constitutive NF-κB activity in HRS cells may well be responsible for the anti-apoptotic phenotype of the HRS cells (Re *et al.,* 2005). There are several mechanisms of activation of NF-κB in cHL which include LMP-1 expression, c-REL amplification, IκBα mutation/deletion and constitutive CD30 signalling (Emmerich *et al.,* 1999;Horie *et al.,* 2002;Joos *et al.,* 2003;Emmerich *et al.,* 2003;Grimm *et al.,* 2005;Osborne *et al.,* 2005;Uchihara *et al.,* 2006). In addition to CD30, CD40 (Gruss *et al.,* 1994) and RANK (Fiumara *et al.,* 2001) are two other members of the TNF receptor family that are expressed on HRS cells and may be contributory in HRS cell survival by activating NF-κB. Future studies on the exact mechanisms of NF-κB activation in cHL are important for both our understanding of disease pathogenesis and the development of novel therapies.

The overexpression of particular cytokines and chemokines in HL has been discussed above. Notably both IL13 and IL13 receptor are expressed by HRS cells and this appears to result in autocrine proliferation (Kapp *et al.,* 1999). Functional experiments from HRS cells lines have confirmed the importance of IL-13 as an autocrine growth factor in HL (Skinnider and Mak 2002;Trieu *et al.,* 2004). There is increasing evidence that loss of function of suppressor of cytokine signalling (SOCS)-1 is involved in the progression of several cancers (Rottapel *et al.,* 2002;Sutherland *et al.,* 2004). More recently a specific function of STAT3, SOCS1 and SOCS3 in the proliferation and survival of cHL cells has been reported (Baus and Pfitzner 2006). They stated that downregulation of STAT3 by siRNA expression decreased cell proliferation and induced apoptosis. Overexpression of SOCS1 and SOCS3 resulted in a proliferation arrest of cells with limited endogenous amount of these negative regulators, but not in cells that already express high amounts of SOCS1 and SOCS3. In addition, SOCS1 mutations have been detected in the HRS cells of 8/19 cHL samples and it has been suggested that this defective tumour suppressor is triggering an oncogenic pathway in HL (Baus and Pfitzner 2006).

The transmembrane receptor Notch 1 when activated initiates expression of multiple genes in the nucleus of the cell. Notch1 is expressed at high levels in the HRS cells of cHL but is absent in normal B-cells. Triggering of Notch 1 in HL derived cell lines initiates a strong proliferation signal indicating that this may be involved in maintaining the HRS cells in vivo (Jundt *et al.,* 2002a).

Deregulation of receptor tyrosine kinases (RTKs) is frequently involved in transformation (Blume-Jensen and Hunter 2001). In a recent study, 6 different RTKs were shown to be expressed and activated in the tumour cells of cHL, particularly in the NS subtype. The authors suggest that the use of tyrosine kinase inhibitors may be of therapeutic use in these cases (Renne *et al.,* 2005).

1.6. The epidemiology of HL

Early epidemiological studies show a bimodal age incidence curve for HL although the age specific incidence peaks occur in different age groups in different situations (MacMahon 1966). Differences in age-specific incidence curves led to the suggestion that at least three epidemiological patterns of HL exist (Correa and O'Conor 1971).

In developing countries the first peak of the bimodal curve occurs in childhood, there is a decrease in incidence in the third decade and a second peak occurs in older age groups. In developed countries, there is a low incidence in childhood, but this increases with age resulting in a pronounced peak in young adulthood (15-34 years). The incidence declines in the fourth decade and in older years a gradual increase or plateau is observed (McKinney *et al.,* 1989;Glaser and Swartz 1990). An intermediate pattern is found in rural areas of developed countries, in central Europe and in the southern United States (Correa and O'Conor 1971;Alexander *et al.,* 1991a).

NSHL has a unimodal age incidence and accounts for the young adult peak seen in developed countries. In early childhood, NSHL is relatively less common. The other subtypes, in particular MCHL show an increasing incidence with age (Glaser and Swartz 1990;Alexander *et al.,* 1991a).

Several hypotheses have been suggested to explain the epidemiological features of HL. MacMahon observed that different age groups 0-14 years, 15-34 years and 50 years and over, had different features of the disease associated with the age at onset. He therefore proposed that HL was a heterogeneous condition and that the three groups had different aetiologies (MacMahon 1966). Epidemiological studies have since confirmed that the risk factors for the young adult and older age groups differ giving rise to a "two disease" hypothesis (Correa and O'Conor 1971;Gutensohn 1982;Alexander *et al.,* 1991a).

Another not mutually exclusive model exists that suggests that HL results from delayed exposure to a common infectious agent. Epidemiological studies have shown an increased risk of developing HL in individuals with a high socio-economic status in childhood (Alexander *et al.,* 1991b). It had been suggested that persons lacking early social contact may have escaped infection with common childhood infections until later in life (Gutensohn and Cole 1977). An extension of this model suggested that HL in childhood in developing countries and young adult cases in developed countries are caused by the same infectious agents(s) (Correa and O'Conor 1971;Gutensohn and Cole 1981). This theory has not been substantiated in subsequent studies (Macfarlane *et al.,* 1995). It was shown that the risk ratios of HL tended to be lower for men who had experienced various common contagious diseases in childhood (Paffenbarger *et al.,* 1977). Another study supports this previous evidence that early exposure to infection protects against HL although adds that a previous history of infectious mononucleosis (IM) increases subsequent risk (Alexander *et al.,* 2000). It has now been shown that children who attend nursery school and day care seem to have a decreased risk of developing young adult HL, most likely due to childhood exposure to common infections, and the subsequent maturation of cellular immunity. This finding supports the delayed infection model of HL aetiology in young adults but also introduces the determinant of age at infection (Chang *et al.,* 2004).

EBV and the epidemiology of HL

Since EBV was associated with a proportion of HL cases it appeared plausible that this may be the infectious agent that would fit the model proposed by Gutensohn and Cole as described above. However, early studies showed that EBV was not associated with young adult cases when compared to other age groups (Jarrett *et al.,* 1991). EBV is therefore not the agent responsible for the young adult incidence peak seen in developed countries. Based on the above epidemiological data it is this group of HL patients which is of particular interest when looking for other infectious aetiologies.

Further studies have clarified the link between EBV status and age at diagnosis. UK data from young and older adults support the idea that these two groups have different aetiologies (Armstrong *et al.,* 1993;Armstrong *et al.,* 1998a). Paediatric cases from different geographical locales were also shown to be EBV-associated, particularly with children under 10 years of age. These data led to the proposal of a "three disease model" which supported MacMahons original theory (MacMahon 1966). The first group comprises paediatric cases (particularly under 10 years) which are EBV-associated and often of the MC subtype; the second group is also EBV-associated and is found in older adults mainly with MC subtype; the third group are non EBV-associated and comprise mostly young adults of the NS subtype.

Infectious mononucleosis and HL

Evidence from two population based studies has help clarify the relationship between EBV and HL (Alexander *et al.,* 2000;Alexander *et al.,* 2003). Previous studies had shown that young adults who had a past history of IM were three times more likely to develop HL (Gutensohn and Cole 1980). The findings from Alexander *et al* confirmed that in general, IM is associated with an increased risk of developing HL. Prior IM also confers an additional risk of developing EBV-associated HL in young adults (Alexander *et al.,* 2000). In these cases it is believed that the IM and EBV-associated disease are causally linked (IARC 1997). One study failed to confirm these findings and reported a dissociation between IM history and EBV-positivity, in that almost all of the cases with a history of IM were EBV-negative (Sleckman *et al.,* 1998). In support of the majority of the literature an association between IM–related EBV infection and the EBV-positive subgroup of HL was subsequently reported as likely in young adults (Hjalgrim *et al.,* 2003).

These data have suggested a "four disease model" (Jarrett 2002). The fourth entity described encompasses EBV-associated cases following delayed infection with EBV. The model consists of three EBV-associated groups and one group that

is non- EBV-associated. The EBV-associated peak in the 15-34 year age group (young adults) represents cases that are associated with delayed exposure to EBV.

1.7. Other viruses and cHL

The exact transforming event in the EBV-negative cases remains unsolved. It has been suggested that a hit-and-run scenario involving EBV is possible. There is persuasive epidemiological evidence that infectious agent(s) are involved in young adult HL in developed countries (section 1.6.). Likely candidates are ubiquitous viruses that infect individuals at older ages in developed countries in comparison with developing countries. Members of the polyomavirus and herpesvirus family fit into this category. No evidence of the polyomaviruses LPV, JC, BK and SV40 viruses was detected in DNA from HL cases (Armstrong *et al.,* 1998b;MacKenzie *et al.,* 2003). Cytomegalovirus, human herpesvirus (HHV)-6, -7 and -8 have not been consistently detected within HL lesions however HHV-6 and -7 are frequently detected at low copy number presumably in bystander cells (Jarrett *et al.,* 1988;Gledhill *et al.,* 1991;Torelli *et al.,* 1991;Khan *et al.,* 1993;Secchiero *et al.,* 1998;Berneman *et al.,* 1998;Cozen *et al.,* 1998;Armstrong *et al.,* 1998b).

The measles virus has also been implicated as a candidate virus in a proportion of HL cases (Benharroch *et al.,* 2003). These results however remain controversial and have not been confirmed (our unpublished data and personal communication R.Küppers).

A tentative link has also been suggested between TT viruses and HL. The hypothesis proposed by the authors leaves many questions unanswered. Further investigative studies on the role of TTVs in HL may help clarify these observations (zur Hausen and de Villiers 2005).

1.8. The aetiology of cHL

The aetiology of cHL is still not completely understood however a combination of both genetic and environmental factors are implicated. A familial predisposition is most evident in monozygotic twins where the co-twin has a risk of developing HL which is 99 times higher than that shown for dizygotic twins (Mack *et al.,* 1995). Ethnic variation is also observed in the incidence of HL which is also supportive of a genetic predisposition (Glaser and Jarrett 1996;Glaser and Hsu 2002). The genetic predisposition appears in part related to immune function in HL with a number of HLA associations having been reported (Hors and Dausset 1983;Taylor *et al.,* 1999;Alexander *et al.,* 2001;Harty *et al.,* 2002). The role of specific HLA alleles and haplotypes is however not clearly defined in HL and because of linkage disequilibrium in the HLA region any association may not be causal.

The most notable environmental factor in the pathogenesis of HL is EBV. Recent studies have shown that genotypic markers in HLA class I and HLA class III are associated with HL susceptibility. Detection of specific microsatellite markers in the HLA class I region is associated with EBV–positive HL. This suggests that the presentation of EBV-derived peptides could be important in the pathogenesis of EBV-positive HL (Diepstra *et al.,* 2005). Further mapping of the HLA regions is essential to identify susceptible loci in HL and the role of antigen presentation in the disease pathogenesis (Poppema 2005).

1.9. Gene expression studies in HL

A number of studies have attempted to determine the overall gene expression profile of HRS cells, the majority of which have used cHL-derived cell lines (Kapp *et al.,* 1999;van den Berg *et al.,* 1999;van den Berg *et al.,* 2000;Kuppers *et al.,* 2003;van den Berg *et al.,* 2003;Schwering *et al.,* 2003a;Schwering *et al.,* 2003b;Janz *et al.,* 2006;Staber *et al.,* 2006) or whole cHL tissue (Devilard *et al.,*

2002;Sanchez-Aguilera *et al.,* 2006). Two studies have used isolated HRS cells to perform gene expression profiling of cHL (Cossman *et al.,* 1999;Karube *et al.,* 2006).

The first study to use single HRS cells for a global gene expression profiling sequenced a large number of expressed sequence tags however the tag profile was a mixture between cHL and NLPHL cells which differ significantly in expression profile (Cossman *et al.,* 1999). The expression profile generated supported a B-cell lineage for HRS cells, but subsequent studies have shed doubt on whether the 'primary tumour' cells were bona fide HRS cells.

The use of the serial analysis of gene expression (SAGE) technique demonstrated the extremely high expression of the chemokine CCL17 from HRS cells and highlighted the possible role it may have in attracting Th2 cells to the surrounding infiltrate (van den Berg *et al.,* 1999). In a subsequent SAGE study the B-cell receptor inducible gene BIC was identified in all subtypes of HL (van den Berg *et al.,* 2003) which led to the investigation and detection of the mature microRNA-155 in HL cell lines and tissue samples (Kluiver *et al.,* 2005).

In comprehensive studies using SAGE and DNA microarrays the loss of the B-lineage specific gene expression program was demonstrated in the HRS cells of cHL (Kuppers *et al.,* 2003;Schwering *et al.,* 2003a;Schwering *et al.,* 2003b). Down regulation of these genes affect multiple pathways in B-cells including BCR signalling. As the B-cell transcriptional activator PAX5 is expressed in most HRS cells and many of its target genes are down regulated the authors checked for mutations of PAX5 in HL cell lines without success. There are a number of mechanisms that may explain the lack of B-cell phenotype in HRS cells; the expression of LMP2A in EBV-positive cHL cases (Portis *et al.,* 2003); the down regulation of B cell transcription factors (Stein *et al.,* 2001;Jundt *et al.,* 2002b); overexpression of the proteins ABF-1 and Id2 which downregulate B-cell specific genes although induce other lineage markers in HRS cells (Mathas *et al.,* 2006)

and gene silencing by methylation of B-cell specific genes (Ushmorov *et al.,* 2006). This lack of B-cell identity may help explain the survival of HRS cells lacking a BCR.

In an additional study using microarrays on 4 HL cell lines, twenty seven genes previously not shown to be expressed in HRS cells were aberrantly expressed, among which were GATA-3, ABF1, EAR3 and Nrf3 (Kuppers *et al.,* 2003). These newly identified genes may be involved in the pathogenesis of HL and could represent novel diagnostic markers that may be considered as therapy targets. In a recent gene expression study of cytokines and chemokines from microdissected primary HRS cells high expression of IL-11 receptor α was detected (Karube *et al.,* 2006). IL-11 receptor is known to activate the JAK/STAT pathway and therefore may be associated with a maligant phenotype.

Further gene expression studies are required using primary HRS cell material to clarify the complex gene expression profile of HRS cells in cHL and perhaps identify candidates involved in the disease pathogenesis.

1.10. Thesis overview

The mechanisms of transformation in HL are still not completely understood however advances over the last decade in micromanipulation of HRS cells and advanced molecular techniques have enabled more understanding and characterisation of this complex lymphoma. Unravelling the transformation process and key elements involved in the disease may lead to new and improved treatment strategies in the future.

Chapter 2 discusses the significance of EBV DNA that is present in the serum of EBV-associated cases of HL and compares this with the non-associated cases and

normal healthy controls. The relevance of the results as a prognostic marker is also evaluated.

Chapter 3 looks at the "hit and run" hypothesis suggested for the role of EBV in the non-EBV-associated cases of the disease. It has also been suggested that integrated fragments of the normally episomal EBV genome may persist in EBV-negative cases. This study aims to tackle these points comprehensively using a combined serologic and molecular approach.

Chapters 4 and 5 describe the use of degenerate PCR strategies for the investigation of known and novel herpesviruses and polyomaviruses that may be present in cHL DNA. Implications for the EBV-negative cases of cHL are discussed. Following on from this Chapter 6 covers molecular methods for virus discovery encompassing those techniques employed in chapters 4 and 5 and dicussing other molecular techniques that may be useful for the investigation of viral contaminants in vaccines. These techniques are relevant for the investigation of possible viral involvement in cHL.

Chapter 7 discusses the SAGE procedure for the investigation of relevant gene expression in cHL using enriched HRS cell populations from primary cHL material. The main aim of this study was to identify genes that are differentially expressed between HRS cells and their 'normal' counterpart. An additional aim was to investigate gene expression differences between EBV-positive and EBV-negative cHL.

Chapter 8 summarises the results obtained in this thesis and discusses the significance of these findings in the current cHL research field. Future experiments are highlighted and novel research strategies explored.

Chapter 2

Detection of Epstein-Barr virus (EBV) genomes in the serum of patients with EBV-associated Hodgkin's disease

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Int J Cancer 1999 Aug; 84(4):442-448

Abstract

DNA from malignant cells is present in the serum/plasma of cancer patients and DNA from this source is amenable to analysis by polymerase chain reaction (PCR). In the present study we evaluated whether Epstein-Barr virus (EBV) DNA is present in the serum of patients with EBV-associated Hodgkin's disease (HD). Using conventional PCR, EBV DNA was detected in serum from 30/33 patients with EBV-associated Hodgkin's disease but only in 6/26 patients with non-EBV-associated disease ($p<0.001$). Samples from healthy individuals were negative and only 5/12 infectious mononucleosis samples were positive. Real-time quantitative PCR was subsequently employed to determine the concentration of EBV DNA present in serum; among positive samples the level ranged from 1 to 705 copies per 125 µl of serum. Post-treatment samples from 5/14 cases with EBV-associated Hodgkin's disease contained detectable EBV DNA; analysis of this small group of cases suggests that positivity in post-treatment samples correlates with risk factors indicative of a poor prognosis. Overall, the results are consistent with the notion that DNA from Reed-Sternberg cells is present in the serum of HD patients, and further suggest that serum EBV should be evaluated as a prognostic marker.

Introduction

For several decades it has been known that patients with cancer and autoimmune disease have increased amounts of DNA in serum and plasma relative to healthy individuals (Leon *et al.,* 1977;Steinman 1979). The polymerase chain reaction (PCR) has been used to analyse DNA sequences from these sources. It has been possible to detect mutations of oncogenes, such as *ras*, in the plasma of cancer patients (Vasioukhin *et al.,* 1994;Anker *et al.,* 1997), and loss of heterozygosity has been demonstrated in serum and plasma samples from patients with head and neck tumours (Nawroz *et al.,* 1996) and small cell lung carcinoma (Chen *et al.,* 1996). The results of the latter analyses indicate that the DNA present in the serum/plasma of cancer patients is largely derived from tumour cells. Such studies suggest that the analysis of serum or plasma DNA may be useful diagnostically and also, more importantly, in the clinical follow-up of cancer patients.

In the present study we addressed whether genomes from the Epstein-Barr virus (EBV) could be detected in the serum of patients from Hodgkin's disease (HD). EBV is detectable in Reed-Sternberg cells, the tumour cells in HD, in around one third of HD cases in western countries and is thought to play a role in disease pathogenesis in these cases (Armstrong *et al.,* 1992;Jarrett *et al.,* 1996). The ability to detect EBV DNA in serum samples from patients with EBV-associated HD could prove useful in both clinical and epidemiological studies.

Materials and Methods

Case and sample selection: Serum samples from 80 patients with HD, 20 patients with clinical symptoms of infectious mononucleosis and IgM antibodies to EBV, and 15 healthy individuals were assayed for the presence of EBV genomes. HD cases were selected on the basis of EBV status of lesions and the availability of pre-treatment serum. The series included samples from 42 cases of EBV-

associated and 38 cases of non-EBV-associated HD. Plasma and serum samples were collected prospectively from a further 6 HD patients in order to compare DNA extracted from these two sources.

DNA extraction: DNA was extracted from 500 µl aliquots of pre-treatment serum or plasma which had been stored at -80°C. DNA extraction was performed using the Blood & Cell Culture DNA Mini Kit (Qiagen, Crawley, UK) but with minor modifications to the manufacturer's instructions. Briefly, 500 µl of G2 lysis buffer and 25 µl of protease were added to the serum sample and the reaction was incubated for 20 minutes at 56°C. Subsequent steps were performed according to the supplier's protocol and samples were resuspended in either 30 µl (for conventional PCR) or 60 µl (for quantitative PCR) H_2O.

Conventional polymerase chain reaction: Initially, EBV PCR was carried out using primers derived from the BamHI W repeat sequence of EBV as previously described (Deacon *et al.,* 1991;Armstrong *et al.,* 1992). Twenty five microlitres of DNA corresponding to 417 µl of serum were assayed in a 50 µl PCR reaction containing 200 µM nucleotides, 1 µM each primer and 1.5 mM $MgCl_2$. A negative water control was assayed after every 2 samples, and a positive control consisting of 100 ng of DNA from the EBV-positive cell line Raji was included in each assay. Thermal cycling was performed on a Perkin Elmer Thermal Cycler (Perkin Elmer Biosystems, Warrington, UK) using the following conditions: initial denaturation at 95°C for 5 min, followed by 40 cycles of ramping to 94 °C over 1 min; 94 °C for 30 sec; cooling to 55 °C over 2 min; 55 °C for 10 sec; heating to 72 °C over 1 min; 72 °C for 30 sec, followed by a final extension step at 72 °C for 7 min. PCR products were analysed by electrophoresis on 8% polyacrylamide gels followed by electroblotting and hybridisation with a ^{32}P-labelled EBV BamHI W probe (Arrand *et al.,* 1981).

Since the primers used in this assay are derived from a region of the EBV genome which is repeated a variable number of times, this assay cannot be used to

quantitate EBV copy number. However, in order to obtain a rough estimate of assay sensitivity, we tested serial 10-fold dilutions of DNA from the Raji cell line. Samples containing 100 ng to 1 pg DNA were assayed; since this cell line is estimated to contain 50 EBV genomes per cell this corresponds to 7.5×10^5 to 7.5 copies of EBV. Six replicates of the highest dilution were assayed.

Post-treatment samples from 16 of the patients with EBV-associated disease were also tested using the conventional PCR assay. Clinical data including age, sex, stage at presentation, prognostic index (Proctor *et al.,* 1991), haemoglobin, and occurrence of relapse were compared for cases scoring negative and positive.

Quantitative polymerase chain reaction: In later experiments we used real-time quantitative PCR to determine the amount of EBV DNA present in serum samples. TaqMan® methodology was used and samples were analysed using an ABI Prism® 7700 Sequence Detection System (Perkin Elmer Biosystems, Warrington, UK). Primers and probe were derived from the EBV polymerase gene and designed using the Primer Express™ software programme (Perkin Elmer Biosystems, Warrington, UK).

5' primer:	AGT CCT TCT TGG CTA GTC TGT TGA C
3' primer:	CTT TGG CGC GGA TCC TC
Probe:	CAT CAA GAA GCT GCT GGC GGC C

In order to optimise the assay and determine the sensitivity, replicates of 10-fold dilutions of Raji DNA containing from 7.5×10^5 to 0.075 genomes per reaction were tested; six replicates of each of the three highest dilutions were assayed. Samples containing human herpesviruses 6, 7 and 8 were analysed to verify specificity.

Samples from 46 cases of HD (26 EBV-associated, 20 non-EBV-associated), were investigated using the quantitative PCR; 25 of these cases had been analysed using the conventional assay. For comparison, samples from an additional 8 cases

of IM and samples from 9 of the healthy controls were assayed. In these experiments, 15 µl of DNA template, corresponding to 125 µl of serum were used. Reactions were performed using TaqMan® Universal PCR Master Mix (Perkin Elmer Biosystems, Warrington, UK) in a final volume of 50 µl with primers and probe at a concentration of 50 nM and 200 nM respectively. Following initial incubations at 50°C for 2 min and 95°C for 10 min, 40 cycles of thermal cycling at 95 °C for 15 sec and 60°C for 60 sec were performed. Replicate dilutions of Raji DNA estimated to contain 7.5 x 10^5 to 7.5 copies were included in each assay and at least one negative control was included for every two samples.

In 16 of the EBV-associated HD cases, the number of EBV-infected cells in the peripheral blood was known (G. Khan, unpublished results). The viral load in these cases was therefore compared with the concentration of EBV genomic sequences in serum.

Determination of EBV status: EBV status of HD lesions had been determined previously using EBV EBER *in situ* hybridisation, but repeat analyses were carried out on selected cases using a commercially available kit (Novacastro, Newcastle-upon-Tyne, UK). The integrity of the RNA in tissue sections from these cases was assessed using fluorescein-conjugated poly dT probes (Novacastro, Newcastle-upon-Tyne, UK), and expression of the EBV LMP-1 protein was investigated using a cocktail of monoclonal antibodies (CS1-4) essentially as described previously (Rowe *et al.,* 1987;Armstrong *et al.,* 1992).

DNase digestion of fractionated samples: In order to determine whether EBV genomes detected in serum from HD patients were present as naked DNA or as virus particles the following experiment was performed. A known positive serum sample was analysed alongside supernatant from the B95-8 cell line, which was included as a positive control for the presence of EBV virions. Samples (500 µl) were clarified by low-speed centrifugation and filtration and virus pelleted by ultracentrifugation at 50,000 rpm for 60 min in a TLA-120.1 rotor in a Beckman

Optima TLX Ultracentrifuge (Beckman Instruments Inc., High Wycombe, UK). The resulting supernatant was divided into 2 aliquots and the pellet resuspended in an equivalent volume of PBS. DNase 1 (Boehringer Mannheim, Lewes, UK) was added to one of the supernatant samples and the resuspended pellet at a final concentration of 1 mg/ml and reactions incubated at room temperature for 1 hour. EDTA was added to stop the reaction and DNA extraction and quantitative PCR were performed as described above.

Comparison of serum and plasma: In experiments designed to compare DNA extracted from serum and plasma, β-globin primers were used. Initially samples from six cases were compared using a conventional PCR assay which amplifies a fragment of 119 bp, essentially as previously described (Saiki *et al.,* 1988). Reaction conditions and controls were as described for the conventional EBV PCR except that only 12.5 µl of the DNA sample were used per reaction. In later experiments, quantitative PCR was performed on samples from 5 of the cases, using the reaction conditions described above for the EBV TaqMan® assay but with the following primers and probe:

5' primer: GGC AAC CCT AAG GTG AAG GC
3' primer: GGT GAG CCA GGC CAT CAC TA
probe: CAT GGC AAG AAA GTG CTC GGT GCC T

Statistical Analysis: Statistical analyses were performed using the package Minitab® (PA, USA).

Results

Analysis of pre-treatment samples using conventional PCR: Serum samples were initially analysed for the presence of EBV genomes using a conventional PCR

assay and the results are shown in Table 1 and Figure 1. This assay is not quantitative but is sensitive because it amplifies a repeat sequence; in dilution experiments we obtained positive results in 2 of 6 samples estimated to contain 7.5 copies of the EBV genome, while lower dilutions were all positive. Serum samples from 30 of the 33 cases of EBV-associated HD were positive in this assay; in 27 of the 30 positive samples amplification products were clearly detectable on ethidium bromide-stained gels. Repeat samples obtained following treatment were available from 2 of the cases which were originally negative; both were positive in the second assay suggesting that failure to detect EBV genomes in the original sample may have occurred for technical reasons. Samples from 6 of the 26 non-EBV-associated cases were also positive; products were visible on ethidium bromide-stained gels in only 4 cases, and in only 1 case (case 3543) was the PCR product visualised on the stained gel of similar intensity to the products from the majority of EBV-associated cases. There was, therefore, a highly significant correlation between the EBV status of lesions and the ability to detect EBV DNA in serum (Pearson product moment correlation coefficient = 0.691, $p<0.001$). Samples from 5 cases of infectious mononucleosis were also positive but none of the 15 samples from healthy individuals gave rise to positive results (Table 1).

Analysis of post-treatment samples using conventional PCR: EBV PCR was performed on post-treatment serum samples from 14 EBV-associated patients who had positive pre-treatment samples. The mean time between samples was 67 weeks and the mean time of follow-up was 90 weeks. Samples from 5 cases were positive following treatment whereas 9 were negative (Table 2). There was no significant difference in the time between samples or the time of clinical follow-up between the 2 groups of cases. Only one patient relapsed (case 1); this patient had detectable EBV genomic sequences in serum at the time of the second sample and was the case with the longest follow-up time. EBV DNA was detectable in serum 6 months before clinical relapse. Cases in which EBV genomic sequences were detectable at the time of the second sample were more likely to have a higher

prognostic index ($p<0.05$, 2 sample *t*-test), a lower haemoglobin ($p<0.01$) and to present at advanced stage.

Quantitative PCR: The above results suggested that a quantitative PCR assay might prove useful in the clinical follow-up of patients and in the differentiation of EBV-associated from non-associated HD cases. We therefore developed a real-time quantitative PCR assay using TaqMan® methodology (Perkin Elmer Biosystems). In this system the PCR reaction contains conventional primers and a probe labelled with reporter and quencher molecules. During specific amplification the probe is cleaved causing separation of reporter and quencher molecules and emission of fluorescence. Fluorescent intensity increases in direct proportion to the amount of specific amplified product and cycle-by-cycle changes are measured. Results are scored during the exponential phase of the reaction when the fluorescence emission is proportional to the input number of template copies. Data are initially expressed as Ct values; the Ct corresponds to the cycle number at which the amplification plot for a given sample crosses the threshold, which is normally set at the point where the fluorescent signal equals ten times the standard deviation of background fluorescence. Ct values were recorded for the serum samples, and dilutions of DNA from the Raji cell line were used to produce a standard curve [log 10 (initial copy number) plotted against Ct value]. This allowed us to extrapolate the concentration of EBV genomes in samples.

In optimisation experiments and in the experimental assays we consistently obtained a positive result when 1 pg of Raji DNA, estimated to contain 7.5 EBV genomes, was included as template. Occasional positive results were obtained ($\leq$2/6 replicates positive) when 0.1 pg Raji DNA (0.75 copies) were assayed but higher dilutions were always negative (data not shown). The assay was clearly reproducible and the above results suggest that it has the ability to detect <7.5 EBV genomes.

Serum samples from 46 cases of HD were analysed using the quantitative assay; duplicate results were available for 36 cases and in these cases the mean value was taken as the final result (Table 3 and Figure 2). Of the 26 samples from EBV-associated cases, 18 were positive with genome concentrations ranging from <7.5 to 705 copies per 125 µl of serum. One sample from an EBV-associated case which scored negative in the conventional assay was positive with a mean copy number of 12 genomes per 125 µl serum. Two of the twenty samples from non-EBV-associated cases gave rise to positive results; 1 of these was estimated to contain only 1 copy of the EBV genome per 125 µl serum. The second sample had a mean EBV copy number of 41 and was derived from the same serum donation as the sample which gave a robust signal in the conventional PCR (case 3543). Three additional samples, derived from non-EBV-associated cases, which gave positive results in the conventional assay were negative in the quantitative assay.

Samples from 4 of the 8 IM patients were positive using the TaqMan® assay and the two highest copy numbers (750 and 1950 genomes/125 µl serum) were obtained from this group. None of the samples from healthy individuals was positive.

For 16 cases of EBV-associated HD, the concentration of EBV genomes in serum was compared with the EBV viral load in peripheral blood mononuclear cells; no correlation was observed between these 2 results.

Cases with discrepant results: Three cases had clear positive staining of Reed-Sternberg cells in the EBER *in situ* hybridisation assay but negative results in the initial serum PCR. All 3 of these cases, however, had detectable EBV DNA in serum at some stage during the course of these experiments - one was positive in the TaqMan® assay and the remaining two had positive post-treatment samples.

The EBER and poly dT assays were repeated and LMP-1 immunohistochemistry performed on the 6 cases which were positive in the serum assay but negative on

in situ hybridisation. Further EBV analyses were negative in all cases, consistent with the original result, however scattered positive bystander cells were visible in all of these cases following EBER *in situ* hybridisation.

Sensitivity to DNase digestion: In order to determine the physical state of the EBV genomes, we subjected a known positive sample to ultracentrifugation and DNase digestion. EBV DNA was not amplified from the pelleted material or from the DNase-digested supernatant, but was detected in the non-digested supernatant sample; this result indicates that the EBV DNA is present as naked DNA and not in virions. EBV sequences were amplified from the DNase-digested, pelleted material from the B95-8 supernatant confirming the presence of virions in this sample.

Comparison of serum and plasma: DNA extracted from plasma and serum was compared in cases from which matched samples were available. Both conventional and quantitative β-globin PCR assays were used. On ethidium bromide-stained gels 6/6 of the serum samples were positive compared to only 2/6 of the plasma samples. The results of the TaqMan® assay (Figure 3) confirmed that the serum samples were the better source of DNA.

Discussion

Our results add to a growing body of data indicating that serum samples from cancer patients provide a useful source of DNA suitable for molecular analyses. In the present study, EBV DNA was detected in the majority of serum samples from patients with EBV-associated HD but was infrequently detected in samples from non-EBV-associated cases. Two assay systems were used to detect viral DNA. Initially we screened samples using conventional PCR and primers derived from a repetitive sequence within the EBV genome. EBV was detected in serum samples from 30/33 (>90%) HD patients with EBV-associated disease but in only 6/26

cases of non-EBV-associated disease; furthermore there was a suggestion that the amount of EBV DNA was lower in the latter cases. We therefore developed a real-time quantitative PCR assay using TaqMan® methodology. The TaqMan® assay was more sensitive than the conventional PCR, however, we used a smaller volume of serum in this analysis and this resulted in a lower overall positivity rate: 18/26 samples from EBV-associated and 2/20 samples from non-associated cases scored positive. Although the amount of EBV DNA detected in the non-EBV-associated cases was low, the TaqMan® assay did not enable us to cleanly separate the two groups of cases on a quantitative basis.

Several published studies have also detected EBV DNA in serum or plasma from individuals with EBV-associated malignancies. Laroche *et al.* (1995) obtained positive results from 9/13 cases of AIDS-related non-Hodgkin's lymphoma using the equivalent of <10µl serum and a PCR assay based on the EBV BamHI W repeat sequence. Mutirangura *et al.* (1998) reported the detection of EBV genomic sequences in serum samples from 13/42 patients with nasopharyngeal carcinoma, an EBV-associated carcinoma, using a conventional PCR assay similar to that used in the present study except that a slightly smaller serum sample (300 µl) was assayed and PCR primers were derived from single copy, rather than repeat, sequence. EBV DNA was detected in serum from 4/7 patients with nasopharyngeal carcinoma and 2/7 HD patients in a study by Fontan *et al.* (1998); in this report technical details of the PCR reaction and the EBV status of the HD patients were not given. Taken together, these results suggest that EBV DNA is present in the serum of patients with a range of EBV-associated malignancies. Using quantitative PCR, we estimated that positive samples from EBV-associated HD cases contained from <7.5 to 705 EBV genomes per 125 µl of serum. The low concentration of EBV DNA present in many cases highlights the importance of assaying a sufficiently large volume of serum, and most probably explains differences in detection rates in these studies. We recommend that DNA extracted from at least 500 µl of serum should be used in future experiments.

We also detected EBV DNA in serum samples from patients with infectious mononucleosis, a benign, usually self-limiting disease associated with primary EBV infection. Approximately half of these samples scored positive and the two samples with the highest EBV genome concentration were from this group. Precise clinical data were not available from this convenience sample but it is possible that EBV copy number relates to disease stage. Previous studies of infectious mononucleosis have detected EBV sequences in the serum of the majority of cases (Yamamoto *et al.,* 1995;Laroche *et al.,* 1995;Fontan *et al.,* 1998) and detection rates have varied in acute and convalescent samples (Yamamoto *et al.,* 1995). In contrast to the situation in HD (see below), EBV DNA in infectious mononucleosis is present in viral particles as well as free DNA (Gan *et al.,* 1994;Yamamoto *et al.,* 1995). In the present study, and in over 200 cases reported in the literature, EBV has not been detected in the serum or plasma of healthy individuals (Yamamoto *et al.,* 1995;Laroche *et al.,* 1995;Fontan *et al.,* 1998;Mutirangura *et al.,* 1998), therefore the detection of EBV in these samples is suggestive of EBV-associated disease.

In EBV-associated HD, EBV genomes are present in Reed-Sternberg cells and in B-cells persistently infected with this virus. EBV infection in both cell types is latent and, although rare Reed-Sternberg cells in a small number of cases may support abortive replication (Pallesen *et al.,* 1991b;Brousset *et al.,* 1993), there is no evidence of productive infection. However, in order to rule out the possibility that serum samples from HD cases contain EBV virions, we investigated whether the EBV DNA was present in pelleted material and was resistant to DNase digestion. The results confirmed that the viral genomes were present as free DNA and not in viral particles.

The correlation between the EBV status of HD lesions and the ability to detect EBV DNA in serum suggests that Reed-Sternberg cells are the most likely source of the viral DNA. However, EBV was detected in serum samples from occasional cases of non-EBV-associated disease. In all of these cases scattered lymphocytes in

lymph node biopsies were EBV-positive, raising the possibility that latently infected B-cells could be the source of the viral DNA. We therefore compared the concentration of EBV DNA in serum with the frequency of EBV-infected cells within peripheral blood mononuclear cells (data not shown). No correlation was observed, suggesting that in most cases the serum EBV is derived from infected Reed-Sternberg cells. In order to substantiate this, it is essential to be able to differentiate between EBV genomes present in Reed-Sternberg cells and those in latently infected B-cells by analysing polymorphic regions of the viral genome. Although we are collecting samples to perform this analysis, we have not identified an informative case. Support for the idea that Reed-Sternberg cells are usually the source of the EBV DNA is provided by Kornacker *et al.* (1999) who recently demonstrated identical immunoglobulin gene rearrangements in biopsy and serum samples from a HD patient (Kornacker *et al.,* 1999).

Post-treatment samples from 14 cases with EBV-associated HD and a positive pre-treatment sample were tested using the conventional assay and 5 were positive. Cases with EBV DNA detectable in post-treatment samples had higher prognostic indices, lower haemoglobin values and presented with stage 3 or 4 disease (Table 2). The single patient who relapsed had EBV DNA detectable in serum 6 months before clinical relapse. The number of cases included in this analysis was small and the time of follow-up short, however the results suggest that this assay may be useful as a prognostic marker and should be investigated as a predictor of relapse. Future studies will include analysis of sequential samples using the quantitative PCR assay.

Previous studies have used DNA extracted from serum or plasma and also crude samples in PCR assays (Chen *et al.,* 1996;Nawroz *et al.,* 1996;Lo *et al.,* 1997). Serum was initially chosen for our analyses because samples were available, and DNA extraction was performed in order to increase the size of the sample to be assayed. We subsequently performed a small comparative study and the results suggested that serum is superior to plasma as a source of DNA.

Overall, our results suggest that EBV DNA is detectable in most, if not all, patients with EBV-associated HD. In contrast, EBV DNA is infrequently found in serum from non-EBV-associated cases and, in this study, was never detected in samples from healthy individuals. This assay could prove useful in epidemiological studies and in the clinical follow-up of patients with EBV-associated HD and other EBV-associated malignancies.

Acknowledgements

This work was supported by the Leukaemia Research Fund as part of a LRF Specialist Programme. AAA is a recipient of a Kay Kendall Leukaemia Fund fellowship. We are indebted to the many clinicians and pathologists who contributed samples to this study and to the Scotland and Newcastle Lymphoma Group, in particular Jo White.

Table 1. Detection of EBV genomes in serum using conventional PCR

Group	Number positive	
	Ethidium bromide-stained gels	Hybridisation
EBV-associated HD	27/33	30/33
Non-EBV-associated HD	4/26	6/26
Infectious mononucleosis	3/12	5/12
Healthy individuals	0/15	0/15

Table 2. Analysis of post-treatment samples using conventional PCR

Case	Age	Sex	Stage	Prognostic Index	Hb	Follow up (weeks)	Interval between samples	Serum EBV PCR
1*	25	M	4	0.8	8.9	152	123	+
2	54	M	4	0.7	9.7	115	115	+
3	25	M	4	0.6	9.9	48	35	+
4	21	M	4	0.6	10.9	41	21	+
5	20	F	3	0.6	10.6	45	45	+
6#	63	M	3	0.6	10.2	112	98	-
7	26	F	3	0.5	11.4	136	136	-
8	19	M	3	0.3	14.0	127	77	-
9	32	M	4	0.3	13.8	103	74	-
10	19	M	3	NK	15.2	NK	66	-
11	35	M	2	0.2	15.3	49	16	-
12	36	M	3	0.7	10.3	48	29	-
13	36	F	2	0.5	11.5	NK	43	-
14	24	M	2	0.6	11.3	99	53	-

Comparison of clinical data between cases with positive and negative post-treatment samples; all cases included in this analysis had positive pre-treatment samples. *Case 1 was the only patient who relapsed; #case 6 died from unrelated causes during follow-up; Hb, haemoglobin; NK, not known.

Table 3. Detection of EBV genomes in serum using TaqMan® PCR[1]

Group	Number	Result			
		Negative	Positive Copies/125µl serum		
			<7.5	7.5-74	>75
EBV-associated HD	26	8	2	12	4
Non-EBV-associated HD	20	18	1	1	0
Infectious mononucleosis	8	4	0	2	2
Healthy individuals	9	9	0	0	0

[1]The majority of samples were analysed in duplicate and mean values were taken as the final result. HD, Hodgkin's disease.

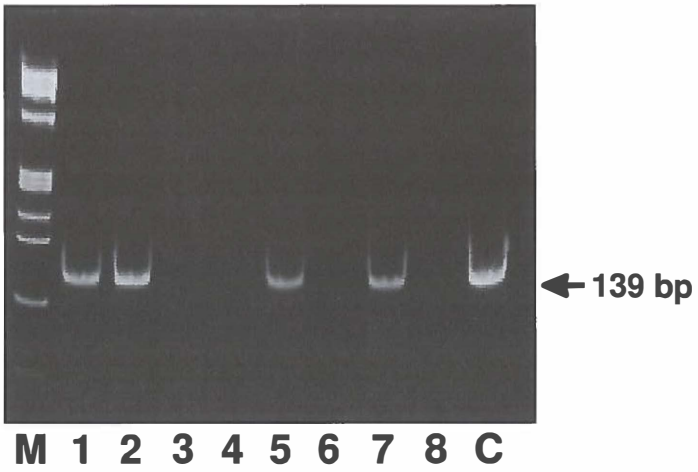

Figure 1. Detection of EBV genomes in serum using conventional PCR. Ethidium bromide-stained gel showing representative results using the conventional PCR assay. M, DNA size marker - Hae III-digested ΦX174 DNA; C, positive control, DNA from Raji cell line; samples were from: EBV-associated Hodgkin's disease, lanes 1, 2, 5 and 7; non-EBV-associated Hodgkin's disease, lanes 4 and 8; healthy individuals, lanes 3 and 6. bp, base pairs.

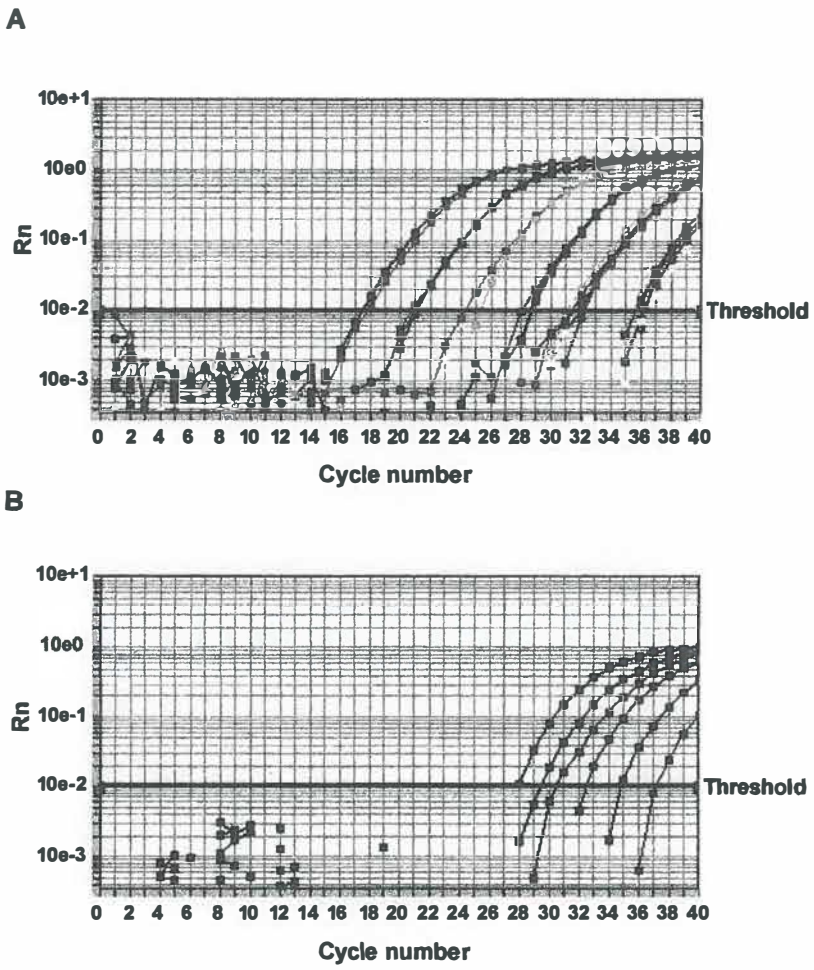

Figure 2. Detection of EBV genomes using TaqMan® PCR. For each sample, the change in fluorescence intensity (dRn) is plotted against cycle number. The result is scored as the cycle number (Ct) at which the amplification plot crosses the threshold. Panel A) analysis of replicates of ten-fold dilutions of DNA from the Raji cell line. Samples were estimated to contain 750,000 to 7.5 copies of the EBV genome. Six replicates of the two highest dilutions and three replicates of lower dilutions were assayed and the Ct values used to construct a standard curve. Panel B) analysis of six representative serum samples from EBV-associated HD cases. Following extrapolation from the standard curve generated from A), these samples were recorded as containing 750, 195, 101, 58, 12 and 3 copies of the EBV genome. Note that the figures given in Table 3 represent mean values.

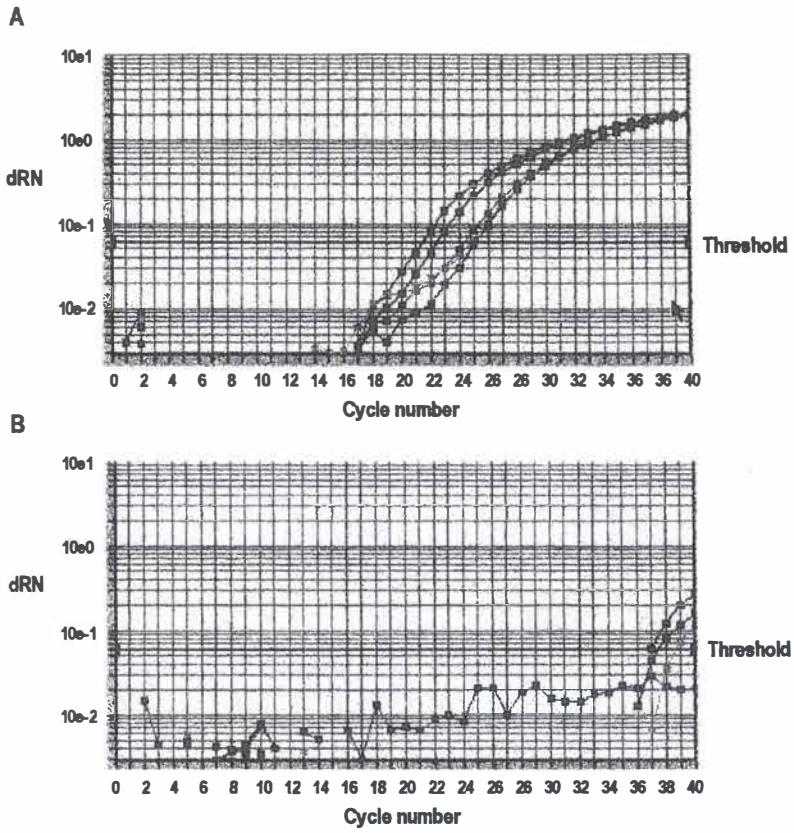

Figure 3. Detection of β-globin DNA sequences in serum and plasma.Analysis of serum and plasma samples from six cases of Hodgkin's disease using TaqMan® PCR. For each sample, the change in fluorescence intensity (dRn) is plotted against cycle number. The result is scored as the cycle number (Ct) at which the amplification plot crosses the threshold. Panel A) DNA extracted from serum and panel B) DNA extracted from plasma. All of the 5 serum samples were positive in this assay whereas only 3 of the plasma samples were positive and these had higher Ct values (lower concentration of β-globin DNA) than the serum samples.

Chapter 3

Hodgkin lymphoma and Epstein-Barr virus (EBV): no evidence to support hit-and-run mechanism in cases classified as non-EBV-associated

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Int J Cancer 2003 May; 104(5):624-630

Abstract

The Epstein-Barr virus (EBV) is associated with a proportion of Hodgkin lymphoma (HL) cases and this association is believed to be causal. The aetiology of cases lacking EBV in the tumour cells (EBV HRS-ve), which make up the majority of cases in western countries, is obscure. It has been suggested that EBV may also cause these tumours by using a hit-and-run mechanism. Support for this idea comes from the finding that most young adult patients, who are likely to have a good immune response to EBV, have EBV HRS-ve HL. We investigated this possibility using a combined serological and molecular approach. Analysis of EBV seroprevalence rates in an epidemiological study of young adult HL revealed that cases with EBV HRS-ve HL were more likely to be EBV seronegative than controls. Furthermore, additional studies clearly showed that some HL patients have never been infected by EBV. Quantitative PCR was used to look for the presence of deleted EBV genomes in a series of adult cases with EBV HRS+ve and EBV HRS– ve HL. Subgenomic fragments were detected in equimolar proportions. This study, therefore, found no evidence to support the idea that a hit-and-run mechanism involving EBV plays a role in the pathogenesis of HL.

Introduction

The Epstein-Barr virus (EBV) is associated with a proportion of cases of Hodgkin lymphoma (HL), and this association is believed to be causal (Jarrett *et al.,* 1996;IARC 1997). In EBV-associated cases, viral genomes are detected in Hodgkin and Reed-Sternberg (HRS) cells, the tumour cells in HL, and EBV latent gene products are expressed (Jarrett 2002). The latter include the EBER RNAs and EBNA1, LMP1 and LMP2 proteins; both LMP1 and 2 are postulated to play a role in disease pathogenesis (Jarrett 2002). EBER *in situ* hybridisation and LMP1 immunohistochemistry are generally used to determine the EBV status of HL tumours, and cases that are positive in these assays are referred to hereafter as EBV-associated or EBV HRS+ve. The aetiology of EBV HRS-ve cases remains obscure.

The epidemiology of HL suggests that it is a heterogeneous condition and the term HL most probably embraces more than one aetiological entity. Consideration of differences in age-specific incidence patterns, along with risk factor data and the distribution of histological subtypes, suggests that HL in children, young adults (15-34 years) and older adults (≥50 years) have different aetiologies (MacMahon 1966). For the young adults, it has been further suggested that delayed exposure to a common childhood pathogen may play a role in disease pathogenesis (Gutensohn and Cole 1980;Gutensohn and Cole 1981).

In developed countries, EBV is associated with around one third of cases, but in developing countries this proportion can be much higher (Jarrett *et al.,* 1996;Glaser and Jarrett 1996;Glaser *et al.,* 1997). Within individual geographical locales, there is also a suggestion that EBV HRS+ve HL is associated with lower socio-economic status and greater material deprivation (Flavell *et al.,* 1999;Grufferman *et al.,* 2001). There are proportionately fewer EBV-associated cases in the young adult age group compared to the childhood and older adult age groups (Armstrong *et al.,* 1998a). The age incidence curve for non-EBV-associated cases in the UK is

unimodal with an incidence peak between the ages of 15-34 years. In contrast, for EBV-associated cases the curve is much flatter with the suggestion of a peak in young adults aged 15-24 years and a second peak in older adults. Males outnumber females among EBV HRS+ve cases, and a significantly higher proportion of mixed cellularity (MCHL) compared to nodular sclerosis (NSHL) cases are EBV-associated (Jarrett *et al.,* 1996;Glaser *et al.,* 1997).

The suggestion that EBV is causally associated with a larger proportion of HL cases but is using a hit-and-run mechanism in cases classified as EBV HRS-ve, has been discussed (Ambinder 2000). Proponents of this idea suggest that EBV HRS-ve patients have a good immune response to EBV, since these individuals are generally young and from socio-economically advantaged backgrounds, and are therefore able to clear the virus from tumour cells. Although there is no example of hit-and-run oncogenesis in a natural setting, such mechanisms are difficult to disprove.

It has also been suggested that integrated fragments of the normally episomal EBV genome may persist in EBV HRS-ve cases. Defective, integrated and rearranged EBV genomes have been detected in sporadic Burkitt's lymphoma providing precedent for this scenario (Razzouk *et al.,* 1996). We previously performed a Southern blot study using large probes spanning the EBV genome but found no evidence that defective or integrated EBV genomes are a frequent occurrence in HL (Byron Cox, data not shown). Similarly, Staratschek-Jox and colleagues found no evidence for persistent integrated fragments in LMP1-negative classical HL using *in situ* hybridisation (Staratschek-Jox *et al.,* 2000). Recently Gan and colleagues adopted a different approach in the investigation of a series of paediatric HL tumours; using PCR they specifically looked for rearranged heterogeneous (het) EBV DNA with a configuration known as WZhet (Gan *et al.,* 2002). In almost one third of cases, including 8/24 cases negative by EBER *in situ* hybridisation, rearranged genomes were detected. This rearrangement juxtaposes *Bam*HI W and Z fragments of the EBV genome and leads to constitutive

expression of the BZLF1 protein. Expression of this immediate early protein can lead to loss of EBV genomes *in vitro* and it has therefore been suggested that infection with rearranged WZhet genomes might lead to loss of episomal DNA from infected tumour cells *in vivo* (Takada *et al.,* 1992;Gan *et al.,* 2002).

Many studies have shown a concordance between expression of EBER RNA and LMP1 protein in HRS cells (Armstrong *et al.,* 1992). In the investigation of >300 HL cases in our laboratory using both EBER *in situ* hybridisation and LMP1 immunohistochemistry we have only rarely identified cases with discordant results (usually EBER-positive, LMP1-negative), and have largely attributed discrepancies to sample quality. However, we recently identified a case of HL, described here, in which HRS cells clearly expressed LMP1 but not EBER (Figure 1). This prompted us to revisit the possibility that deleted or rearranged EBV genomes may be present in HRS cells in HL and that EBV may use a hit-and-run mechanism in HL. Using serological methods, we looked for evidence that EBV-seronegative individuals are under-represented among HL patients and using quantitative PCR (qPCR) we have looked for evidence of defective and rearranged EBV genomes.

Materials and Methods

Serological study: Serum samples were collected as part of a UK population-based, case-control study of HL in young adults (Alexander *et al.,* 2000;Alexander *et al.,* 2001). Cases were aged 16-24 years at diagnosis and controls were matched on gender, year of birth and county of residence. EBV status of tumours was determined for most cases but only those with confirmed EBV HRS-ve HL are included in our study.

Antibodies against EBV viral capsid antigen (VCA) and early antigen (EA) were detected using indirect immunofluorescence assays (IFAs) as previously described (Henle and Henle 1973;Long *et al.,* 1974). Serum samples were screened at an

initial dilution of 1:10 and positive samples were diluted 2-fold until an end titer (reciprocal of serum dilution at which specific immunofluorescence was last seen) was reached. Results of a comparison between EBV HRS+ve and HRS-ve cases have been reported previously (Alexander *et al.,* 2001). In order to confirm that samples negative in the IFAs were truly negative, available serum samples, diluted 1:100, were screened for IgG antibodies to EBV nuclear antigens (EBNA) using a commercially available kit (ETI-EBNA-G, Diasorin s.r.l., Saluggia, Italy).

Statistical analyses compared results from the EBV HRS-ve HL cases with controls in three ways. First, the proportion of EBV seronegative cases in the two groups was compared. Secondly, results of EBV VCA and EA assays were grouped into 4 categories of approximately equal size according to antibody titre (Table 1). The grouped data were then subjected to contingency table analysis with and without stratification for sex. Thirdly, antibody titres were compared using the Wilcoxon test.

EBER *in situ* hybridisation and LMP1 immunohistochemistry: Both assays were carried out on sections of routinely fixed, paraffin-embedded material. The EBER *in situ* hybridisation assay utilised a commercially available probe (Vector Laboratories, Peterborough, UK) and hybridisation kit (Dako Ltd., Cambridgeshire, UK). Following antigen retrieval, the CS1-4 cocktail of monoclonal antibodies (Dako Ltd.) was used to detect the presence of LMP1. Positive reactivity was detected using the ABC technique incorporating DAB as the chromogenic substrate (Vector Laboratories).

PCR and nucleotide sequencing: qPCR assays detecting sequences spanning the EBV genome were used to determine whether a) we could detect deletions in EBV genomes present in EBV HRS+ve tumours and b) we could detect remnants of EBV genomes in EBV HRS-ve tumours. DNA was extracted from diagnostic biopsies from 30 cases of classical HL (CHL) [Table 2] using proteinase K digestion and extraction with organic solvents (Trainor *et al.,* 1982). qPCR was

performed using TaqMan® methodology (Applied Biosystems, Warrington, UK) and the primer probe sets listed in Table 3. Repeat samples from 8 cases were investigated. Reactions were performed in a total volume of 50 µl and included 1 µg of DNA, each primer at 50 nM (except *Bam*HI e3 assay where 3' primer was 300 nM), probe at 200 nM, and TaqMan® Universal PCR Mastermix (Applied Biosystems). For three cases (13, 17 and 21) there was insufficient DNA to perform analysis using the *Bam*HI H assay. Amplification and analysis were performed on an ABI PRISM™ 7700 Sequence Detection System (Applied Biosystems) using the default thermal cycling parameters for 40 cycles (Gallagher *et al.,* 1999). Dilutions of DNA from the Raji cell line were used as a positive control in all assays except the *Bam*HI e3 assay; EBV genomes in the Raji cell line are deleted in this region and therefore DNA from the JiJoye cell line was used as the positive control. Negative controls, containing water instead of template DNA, were included in a 1:2 ratio with test samples.

An additional TaqMan™ was designed specifically to detect rearranged EBV genomes of the WZhet configuration. Primers and probe (minor groove binding, Applied Biosystems) are shown in Table 3 and reaction conditions were as above. DNA from the cell line P3HR1 clone 5, kindly provided by John Sixbey, was used as a positive control.

Conventional PCR was carried out using primers derived from the EBV LMP1 gene (Table 3) and primers spanning the WZhet rearrangement (Gan *et al.,* 2002). A Taq DNA Polymerase kit (Qiagen, Crawley, UK) with buffer containing 1.5 mM $MgCl_2$ and 200 µM nucleotides (Amersham Pharmacia Biotech, Buckinghamshire, UK) was used and thermal cycling conditions were as previously described (Gallagher *et al.,* 1999). Positive and negative controls were as described above. Products from the LMP1 PCR were sequenced, either directly or following cloning using a PCR®4 TOPO TA cloning kit (Invitrogen, Groningen, The Netherlands). Cycle sequencing was performed using the Big Dye™ Terminator kit v2.0 (Applied

Biosystems) followed by analysis on an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems). Products from the conventional WZhet PCR were subjected to electrophoresis on 8% polyacrylamide gels followed by electroblotting and hybridisation with a *Bam*HI W probe, labelled with [α-^{32}P]dCTP using a rediprime™ kit (Amersham Pharmacia Biotech).

Case 31: Case 31 was a 37 year old, female patient with NSHL. Both fresh, viably stored and fixed material were available from her diagnostic biopsy. Sections of the fixed material were subjected to EBER *in situ* hybridisation and LMP1 immunohistochemistry and used in PCR experiments. Microdissection was also performed in order to obtain regions of paraffin-embedded sections containing clusters of HRS cells. CD15-labelled MiniMACS magnetic beads (Miltenyi Biotec Ltd., Surrey, UK) were used to enrich HRS cells from a cell suspension prepared from the fresh sample; the level of enrichment was assessed by flow cytometry on an EPICS Elite flow cytometer (Coulter Electronics Ltd., High Wycombe, UK). qPCR assays for EBV and β-globin were carried out on: DNA extracted from the fresh biopsy; lysates of cell fractions obtained in the MiniMACS experiment; extracts obtained from paraffin sections by boiling for 30 minutes in lysis buffer (Tris HCl 10 mM, NP40 0.45% v/v, Tween 20 0.45% v/v); and similarly prepared extracts of HRS cells obtained by microdissection.

Results

Serological analysis: Results were available from 72 cases of EBV HRS-ve HL and 103 controls. We first compared the proportion of seronegative samples in the 2 groups. Negative results for both EBV VCA and EA antibodies were obtained for 16 of 72 cases, including 13 of 64 classical HL cases and 12 of 103 controls. "All HL" and classical HL cases were therefore more likely to be EBV-seronegative than controls and differences are statistically significant (p = 0.02 and 0.05, respectively, using Fisher's exact). Differences are focused in the younger age

group (<20 years), the more affluent, firstborn children and subjects with just one sibling and are evident in both sexes.

Sera giving negative results in both IFA assays were subsequently screened for antibodies to EBNA; all of the 14 HL and 11 control samples tested were negative in this assay, providing robust evidence of EBV seronegativity.

The contingency table analysis of the IFA results grouped by titre shows similar distributions for the EBV HRS-ve cases and controls, especially for EA (Pearson's chi-square values exceeded 0.15 and linear trend chi-square p-values exceeded 0.6) [Table 1]. Similarities persist following stratification by sex. The Mann-Whitney tests, examining actual titres, indicate slightly lower VCA and EA levels in the controls but this does not approach statistical significance (p=0.53 and 0.85, respectively).

PCR and sequence analysis: DNA extracted from tumour samples from 30 HL cases was investigated using 7 EBV qPCR assays. Results are expressed as Ct values, the cycle number at which the amplification plot crosses a threshold value, which are inversely proportional to the amount of target DNA. A Ct value of 40 indicates a negative result. Results for the 30 cases were ranked according to the Ct value obtained in the EBV *pol* assay, the qPCR assay routinely used for EBV detection in our laboratory. Results for the *pol* assay were then compared with results from each of the other assays in a pairwise fashion and outliers were identified (Figure 2). This method of comparison is subject to fewer sources of error than the comparison of absolute copy numbers, and gave consistent results when repeat sampling of individual samples was performed.

Generally there was a good correlation between the results of the various EBV assays, although correlation was less good at low viral copy numbers (Ct values above 35) where chance-sampling differences are likely. The EBER and *Bam*HI e3 assays were less sensitive than the other tests, giving higher Ct values; this

most probably reflects the length and GC content of the amplimer sequences. The *Bam*HI W assay detects a repetitive sequence of variable copy number and, as anticipated, this assay was marginally more sensitive than other assays. There was no absolutely clear value that distinguished EBV HRS+ve and HRS-ve cases, although all EBV HRS+ve cases had Cts below 30 in the *pol* assay and gave positive results in all of the assays.

In the ori P assay, one sample (case 3) had a higher than expected Ct, suggesting that the amplimer sequence in this case was deleted or contained a sequence polymorphism (Figure 2b). There was insufficient DNA from this case to allow further exploration of these possibilities. Several samples (from cases 1, 2, 5, 12 and 13) gave unexpectedly high Ct values in the LMP1 assay (Figure 2f). Conventional primers were designed to amplify a region of the LMP1 gene surrounding the qPCR amplimer; samples from cases 1, 2, 5 and 12 were amplified and PCR products sequenced. Identical sequence polymorphisms, involving one nucleotide in the 5' primer and two nucleotides in the 3' primer (Table 3), were identified in the qPCR sequence. These polymorphisms have been previously reported in LMP1 sequences deposited in public databases. A modified 3' primer was designed (Table 3), incorporating two of the base substitutions, and the samples were reanalysed; the polymorphism in the 5' primer is at position 3 and is therefore less likely to affect PCR efficiency. Ct values in the modified assay were all lower than in the original assay confirming that the initial results were due to polymorphic, and not deleted, sequences.

The presence of rearranged EBV genomes was investigated using TaqMan™ and conventional PCR. DNA from the positive control, P3HR1 clone 5, gave rise to clear positive results in both assays but none of the 30 adult HL samples was positive, even following long exposures of Southern blots (Figure 3).

Case 31: EBER *in situ* hybridisation, performed on the diagnostic biopsy, showed positive staining of scattered lymphocytes and also a cluster of cells that appeared

to reside within a germinal centre (Figure 1). HRS cells were negative in this assay. In contrast, there was clear staining of the HRS cells following immunohistochemical staining using the LMP1 antibodies (Figure 1). Both assays gave identical results on repeat staining. To confirm that the HRS cells were EBV-positive, we enriched HRS cells using CD15 MiniMACS magnetic beads and examined the starting sample and CD15-enriched fraction using the *pol* and β-globin qPCR assays. Following normalisation for levels of the β-globin gene, the CD15-enriched fraction contained 7.2-fold more EBV genomes than the starting sample, consistent with the presence of EBV genomes within HRS cells. Similarly, HRS cell clusters microdissected from paraffin sections were positive in the EBV *pol* qPCR.

We next attempted to determine whether the lack of EBER expression by HRS cells was due to a defective EBV genome or downregulation at the transcriptional level. qPCR assays for EBER and *pol* were performed on a small sample of DNA from the CD15-enriched cell fraction and DNA extracted from paraffin sections. Although Ct values obtained in the EBER assay were high (36.3 and 37 respectively) compared to those obtained using the *pol* assay (29.3 and 30.3, respectively) these results were consistent with those of other samples in the ranked comparison.

Discussion

EBV is thought to play a causative role in the pathogenesis of EBV HRS+ve HL. The aetiology of cases lacking EBV genomes in HRS cells is poorly understood, but it has been suggested that EBV may use a hit-and-run mechanism in these cases. If EBV is truly responsible for all HL, then all cases should show evidence of infection by EBV. We investigated this possibility by performing EBV VCA and EA serology on a population-based series of young adult cases. EBV seronegative cases of HL were identified and there was no evidence that seronegative subjects

were under-represented in EBV HRS-ve HL. In contrast, EBV HRS-ve cases were significantly more likely to be EBV seronegative than controls; we have previously reported that these cases had experienced fewer childhood infections than their matched controls and the above finding provides further evidence of lack of exposure to infectious agents during childhood (Alexander *et al.,* 2001). For EBV seropositive subjects, antibody titres were similar in cases and controls. Serological assays for VCA and EA provide good tests for EBV infection; however, as an additional measure we investigated samples that were negative for VCA and EA antibodies using an EBNA ELISA. All of the IFA-negative samples were also negative for anti-EBNA IgG.

There are rare examples of individuals who are EBV-seronegative but from whom virus can be recovered from peripheral blood. We therefore looked for additional evidence for lack of EBV infection in HL. In a recent study, performed in our laboratory, we evaluated the frequency of EBV-infected cells in the peripheral blood of HL cases. Three patients were EBV seronegative and had no detectable EBV in any of 20 aliquots of $2x10^5$ peripheral blood mononuclear cells (Khan *et al.,* 2002). In an additional study we failed to detect EBV-specific cytotoxic T-cell responses in an EBV seronegative HL patient (Chapman *et al.,* 2001). These studies provide robust evidence that some HL patients have never been infected by EBV. Thus, EBV is not the sole aetiological agent in HL.

We investigated a HL biopsy in which the tumour cells expressed LMP1 protein but were EBER negative. Further analysis using qPCR suggested that the lack of EBER RNA expression was not due to deletion of the EBER gene in this case. Expression of LMP1 is essential for transformation of B-cells by EBV, and LMP1 is postulated to play a crucial role in the survival of EBV+ve HRS cells by mimicking a constitutively active CD40 receptor (Kieff and Rickinson 2001;Jarrett 2002). The function of EBER RNAs is less clear; EBER expression is not required for B-cell transformation (Swaminathan *et al.,* 1991), but recent studies suggest that EBER RNAs may play some role in oncogenesis (Komano *et al.,* 1999;Yamamoto *et al.,*

2000). Analysis of case 31 indicates that continued expression of EBER transcripts is not necessary for maintenance of the transformed phenotype in HRS cells. Although expression of EBER RNAs is generally considered a hallmark of latent EBV infection, lack of EBER expression has been described in EBV-associated hepatocellular carcinoma and EBV PCR-positive breast cancers (Sugawara *et al.,* 1999;Bonnet *et al.,* 1999).

To search for defective EBV genomes, we assayed a series of 30 HL samples using seven qPCR assays spanning the EBV genome. In this type of analysis, Southern blotting has the advantage that probes covering the entire genome can be used and restriction fragment length polymorphisms identified; however, this technique is often at the limits of sensitivity in the investigation of EBV genomes in HL. PCR has the advantage of sensitivity and the TaqMan™ assays used in the study facilitated relative quantitation of subgenomic fragments. Results were ranked according to the values obtained in the EBV *pol* gene assay and then comparisons with the results for the remaining six assays were performed in a pairwise fashion (Figure 2). EBV genomes with polymorphisms in the LMP1 gene were clearly identified, thus validating this approach and method of analysis. A single sample with a deletion or polymorphism in the ori P region was also identified but further analysis of this case was not possible. Comparison of results for the other samples suggested that each part of the genome was present at approximately equimolar concentrations. There was no evidence of either deletion or retention of parts of the genome. We further investigated whether rearranged EBV genomes could be detected in adult HL samples using both TaqMan™ and conventional PCR. No positive results were obtained, suggesting that loss of EBV genomes from HRS cells following infection by EBV virions with rearranged genomes is not a frequent event in the pathogenesis of adult HL.

The aim of this study was to investigate the hypothesis that EBV is causatively associated with all, or most, HL cases. The results provide good evidence that some patients with HL have never been infected with EBV, thus EBV cannot be

associated with all cases. Although we did identify a case of HL in which the tumour cells were LMP1-positive but EBER-negative, we found no evidence of defective EBV genomes in this case. Analysis of a further 30 cases provided no evidence that defective EBV genomes are a feature of HL. Overall, this study provides no evidence to support a hit-and-run hypothesis for EBV in HL.

Acknowledgements

We thank the many pathologists and clinicians, in particular Stuart Gardiner and John Murphy, who contributed sample material to this study and Nigel Kirkham for help with photography. This work was supported by a LRF Specialist Programme Grant.

Table 1. Serological results

EBV VCA			EBV EA		
Grouped level	HL Number(%)	Controls Number(%)	Grouped level	HL Number(%)	Controls Number(%)
≤40	18 (25%)	20 (19%)	Negative	28 (39%)	40 (39%)
80 - 320	8 (11%)	25 (24%)	10, 20	18 (25%)	22 (21%)
640, 1280	26 (36%)	33 (32%)	40	10 (14%)	17 (16.5%)
>1280	20 (28%)	25 (24%)	≥80	16 (22%)	24 (23%)

EBV VCA, Epstein-Barr virus capsid antigen; EBV EA, Epstein-Barr virus early antigen; HL, Hodgkin lymphoma.

Table 2. Cases included in qPCR analyses

Case no	Age	Diagnosis	EBV status
1	42	NSHL	+
2	65	MCHL	+
3	21	NSHL	+
4	50	LRCHL	+
5	29	NSHL	+
6	28	CHL NOS	+
7	22	NSHL	+
8	33	CHL NOS	+
9	25	NSHL	ND
10	31	NSHL	+
11	36	NSHL	+
12	63	NSHL	+
13	41	NSHL	+
14	15	MCHL	+
15	43	NSHL	NR
16	52	NSHL	-
17	38	NSHL	ND
18	16	NSHL	-
19	63	NSHL	-
20	15	NSHL4	-
21	43	MCHL	-
22	30	CHL NOS	-
23	33	NSHL 4	-
24	41	NSHL 4	-
25	21	NSHL 4	-
26	25	MCHL 2	-
27	29	NSHL 4	ND
28	59	NSHL 4	-
29	19	NSHL 4	-
30	15	NSHL 4	-

NSHL, nodular sclerosis Hodgkin lymphoma; MCHL, mixed cellularity Hodgkin lymphoma; LRCHL, lymphocyte rich classical Hodgkin lymphoma; CHL NOS, classical Hodgkin lymphoma not otherwise specified. EBV status refers to the presence (+) or absence (-) of EBV in Hodgkin Reed-Sternberg cells as assessed by EBER *in situ* hybridisation or LMP1 immunohistochemistry. ND, not done, sections not available; NR, no result, sections not suitable for scoring.

Table 3. Primers and probes used in PCR analyses

PCR assay Amplimer positio		
EBV EBER	5'primer	AGGACCTACGCTGCCCTAGAG
6629-6795	Probe	AGCCACACACGTCTCCTCCCTAGCAAA
	3'primer	AACCACAGACACCGTCCTCAC
EBV ori P	5'primer	AGGCGCAAGTGTGTGTAATTTGT
8104-8167	Probe	CTCCAGATCGCAGCAATCGCGC
	3'primer	GGGCGGGCCAAGATAGG
EBV *Bam*HI W	5'primer	CCCCTGGTATAAAGTGGTCCTG
14345-14419	Probe	AGCTATTTCTGGTCGCATCAGAGCGC
	3'primer	CCCTCTTACATTTGTGTGGACTCC
EBV *Bam*HI H	5'primer	AGTCGTGTGCATGGAAATGG
54424-54498	Probe	ACCCTGCATCCTGTGTTGGAGCTAGC
	3'primer	CGAAAGGCGGAGAGGTGTT
EBV *Bam*HI e3	5'primer	TGTCCAGTTCCCTTCTCCCA
101592-101664	Probe	CCTGTACACCCCGACCCAAAGGG
	3'primer	GGTAACTAGAAATCTGAATGCCATTGA
EBV *pol*	5'primer	AGTCCTTCTTGGCTAGTCTGTTGAC
154828-154738	Probe	CATCAAGAAGCTGCTGGCGGCC
	3'primer	CTTTGGCGCGGATCCTC
EBV LMP1	5'primer	TCATCGGTAGCTTGTTGAGGGT
168692-168760	Probe	ACCACCACGATGACTCCCTCCCG

	3'primer	TGGACAACGACACAGTGATGAA
EBV LMP1 variant	3'primer	TGGACCACGACACACTGATGAA
EBV WZhet	5'primer	GCAGCAGACATTCATCATTTAGAAA
	Probe	CAGTGGTCCCCCTCC*
	3'primer	CAGACAGCAGGCAATTGTCAGT
EBV LMP1 conventional	5' primer	TGGAGTTAGAGTCAGATTCATGGCC
	3' primer	TGGTTGATCTCCTTTGGCTCCT
β-globin	5'primer	GGCAACCCTAAGGTGAAGGC
	Probe	CATGGCAAGAAAGTGCTCGGTGCCT
	3'primer	GGTGAGCCAGGCCATCACTA

Amplimer co-ordinates refer to the complete EBV genomic sequence: GenBank accession number V01555. *Minor groove binding probe.

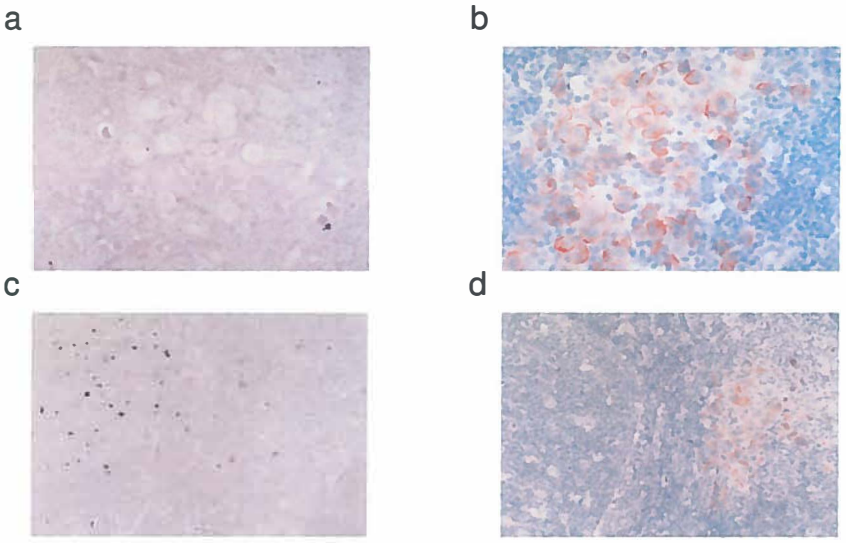

Figure 1. *In situ* analysis of case 31. Panels a and b: High power view of a cluster of Hodgkin and Reed-Sternberg (HRS) cells stained using EBER *in situ* hybridisation and LMP1 immunohistochemistry, respectively. No specific staining is observed in panel a, whereas positively staining HRS cells are observed in panel b. Panel c: Low power view showing positive staining of a cluster of small cells in the EBER *in situ* hybridisation assay. Panel d: Similar area of section, stained using LMP1 immunohistochemistry, showing positive staining of HRS cells but lack of staining of small cells.

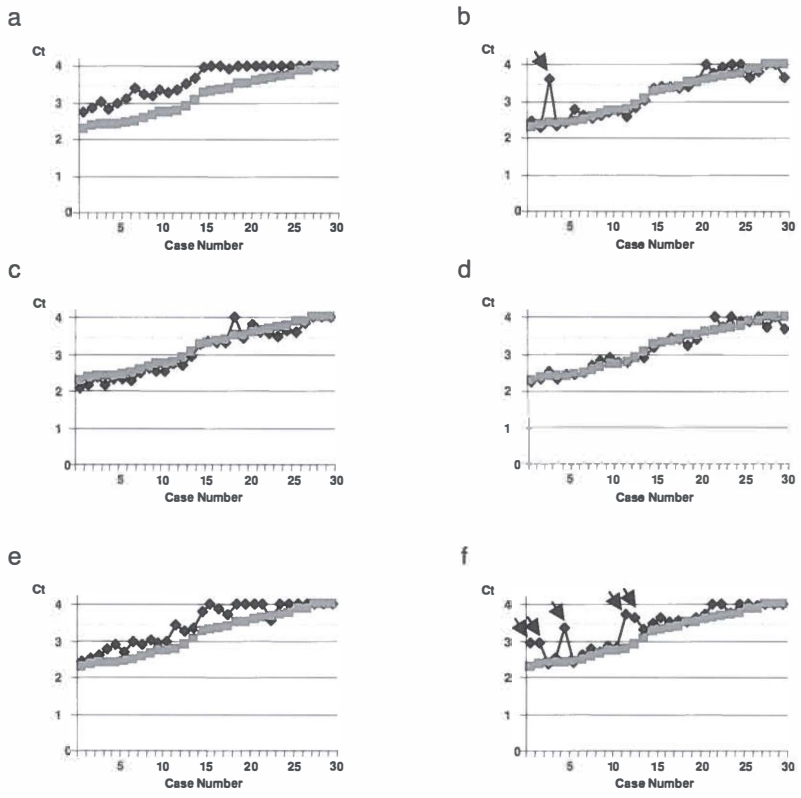

Figure 2. Analysis of qPCR results. qPCR results were ranked according to the Ct values obtained in the EBV *pol* qPCR assay. The result for each sample in this assay (square symbols) was compared with the result in each of the other assays (diamond symbols): panel a, EBER; panel b, ori P; panel c, *Bam*HI W; panel d, *Bam*HI H; panel e, *Bam*HI e3; panel f, LMP1. Samples with values outside the expected range are indicated with arrows.

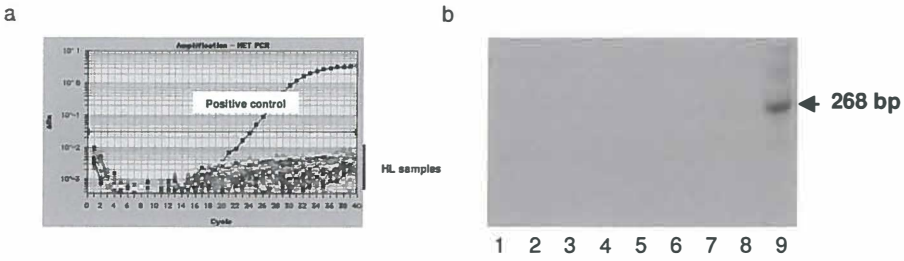

Figure 3. Rearranged EBV genomes are not detected in adult HL samples. Panel a: TaqMan™ analysis of HL samples for the presence of rearranged EBV genomes with the WZhet configuration. The positive control is giving rise to a clear positive result with the amplification plot crossing the threshold after 24 cycles of amplification. Amplification plots from the HL samples do not reach the threshold value. Panel b: Conventional PCR analysis followed by Southern blotting and hybridisation to an EBV *Bam*HI W probe. Lane 1, case 22; lane 2, negative control; lane 3, case 7; lane 4; case 8; lane 5, negative control; lane 6, case 23; lane 7, case 24; lane 8, negative control, lane 9; positive control. The positive control is giving rise to a positively hybridising fragment of 268 bp (base pairs), whereas all the HL samples are negative.

Chapter 4

Viruses and Hodgkin's disease: no evidence of novel herpesviruses in non-EBV-associated lesions

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Int J Cancer 2002 Sept; 101(3):259-264

Abstract

The Epstein-Barr virus (EBV) is associated with a proportion of cases of Hodgkin's disease (HD) and this association is believed to be causal. Epidemiological studies suggest that an infectious agent is involved in the aetiology of young adult HD, however, cases in this age group are less likely to have EBV-associated disease than cases diagnosed in early childhood or older adult years. Molecular studies have failed to find a consistent association between HD and other candidate viruses, and the aetiology of non-EBV-associated cases remains obscure. We looked for evidence of herpesvirus infection in samples of non-EBV-associated HD using a highly sensitive, degenerate PCR assay. Despite exhaustive sequence analysis of PCR products, no novel herpesviruses were identified. These results suggest that it is extremely unlikely that a novel herpesvirus is involved in the pathogenesis of non-EBV-associated HD.

Introduction

The lymphotropic herpesvirus Epstein-Barr virus (EBV) is associated with a proportion of cases of Hodgkin's disease (HD) and this association is believed to be causal (IARC 1997;Jarrett 1998;Jarrett 2002). In EBV-associated HD (EBV+HD) the Reed-Sternberg cells, the tumour cells of HD, are infected by EBV and viral genes are expressed (Wu *et al.,* 1990;Pallesen *et al.,* 1991a;Jarrett 1998). Although most Reed-Sternberg cells are derived from germinal centre B-cells (Kuppers and Rajewsky 1998), they do not express B-cell receptor (BCR) complexes and it is not clear how they escape apoptosis, the normal fate of mature B-cells lacking BCRs. It is postulated that, in EBV+HD, expression of the EBV latent membrane proteins 1 and 2A facilitates survival of Reed-Sternberg cells by mimicking CD40 and BCR signalling respectively (Jarrett 2002).

HD has an unusual age distribution with geographical and ethnic variation in age-specific incidence patterns (MacMahon 1966;Correa and O'Conor 1971;Macfarlane *et al.,* 1995;Glaser and Jarrett 1996). Classically the incidence is described as bimodal with incidence peaks occurring in childhood and older adults (>50 years) in developing countries, and in young adults (15-34 years) and older adults in developed countries. In practice, variations on these patterns are observed (Macfarlane *et al.,* 1995;Glaser and Jarrett 1996). The clinicopathological features of HD differ by age at diagnosis (MacMahon 1966;Glaser and Jarrett 1996). The vast majority of young adult cases have nodular sclerosing HD (HDNS) whereas in older adults and children the mixed cellularity subtype (HDMC) is relatively more common (MacMahon 1966;Glaser 1987;McKinney *et al.,* 1989;Glaser and Jarrett 1996). Male cases predominate in childhood and older adult age groups whereas there is no significant gender bias among young adults cases (Glaser and Jarrett 1996). In 1966 MacMahon (MacMahon 1966) suggested that HD in different age groups has different aetiologies and subsequent data provide support for this hypothesis. Epidemiological studies have consistently found that young adult HD cases have risk factors suggesting a high standard of living in early childhood

(Gutensohn and Cole 1980;Gutensohn 1982;Glaser 1987;McKinney *et al.,* 1989;Alexander *et al.,* 1991a). This has led to the suggestion that HD in this age group is caused by delayed exposure to a common infectious agent (Gutensohn and Cole 1980). In support of this model, individuals with HD diagnosed in young adult life report fewer childhood infections than control groups (Paffenbarger *et al.,* 1977).

Many studies have examined the relationship between EBV-association and HD occurring in different geographical locales and patient subgroups. In the UK around one third of cases are EBV-associated whereas in developing countries this proportion is generally much higher (Jarrett *et al.,* 1996;Glaser *et al.,* 1997;Armstrong *et al.,* 1998a;Jarrett 2002) A greater proportion of HDMC cases are EBV-associated compared to HDNS cases and this difference has been statistically significant in almost every study (Pallesen *et al.,* 1991a;Jarrett *et al.,* 1996;Glaser and Jarrett 1996). Among EBV+HD cases, males outnumber females whereas gender differences are not significant for non-EBV-associated cases (Glaser *et al.,* 1997;Alexander *et al.,* 2000). EBV status also varies significantly with age; most cases diagnosed in early childhood and older adult years are EBV+HD whereas only a minority (~25% in the UK) of young adults cases are EBV-associated (Jarrett *et al.,* 1991;Jarrett *et al.,* 1996;Glaser *et al.,* 1997;Jarrett 2002). Recent evidence suggests that EBV+HD occurring in the young adult age group is frequently associated with late exposure to EBV (Alexander *et al.,* 2000;Alexander *et al.,* 2001;Jarrett 2002).

Based on the above data, we have proposed a four-disease model of HD that defines subgroups of cases on the basis of age at diagnosis and EBV status (Jarrett 2002). Three groups of EBV+HD cases are recognised along with a single group of non-EBV-associated cases; the latter group has a unimodal age distribution with a pronounced peak in the young adult age group and a tail extending into the older adult age group. Young adult HDNS cases typify this

group but older cases and HDMC cases are also represented. The aetiology of these cases is the subject of the present study.

Few epidemiological studies of HD have included an analysis of the EBV status of lesions and therefore hypotheses of aetiology for non-EBV-associated cases are largely based on consideration of young adult cases. One case-control study of young adult HD, which did classify cases by EBV status, provides support for the idea that non-EBV-associated cases have experienced fewer childhood infectious illnesses than controls (Alexander *et al.,* 2000). This suggests that late exposure to a common infectious agent may be involved in the pathogenesis of these cases. We, and others, have looked for genomes of lymphotropic and/or oncogenic viruses other than EBV in HD biopsies with negative results (Jarrett *et al.,* 1988;Khan *et al.,* 1993;Secchiero *et al.,* 1998;Berneman *et al.,* 1998;Armstrong *et al.,* 1998b) and Mackenzie et al., submitted. In particular, there is no evidence for involvement of known members of the herpesvirus family including human cytomegalovirus (HCMV), human herpesvirus (HHV) -6, HHV-7 and HHV-8. Given the epidemiological findings, candidate viruses should be prevalent in the population and living conditions should influence age of exposure. Members of the herpesvirus family fit these criteria and we therefore reasoned that a hitherto unknown herpesvirus might be involved in HD pathogenesis. To test this hypothesis we used a degenerate PCR strategy to search for herpesviruses in samples of non-EBV-associated HD. We have previously used this methodology to look for herpesvirus genomes in clinical samples from human and animal species and have shown that our assay is able to detect a wide range of herpesvirus genomes, (MacKenzie *et al.,* 2001) and unpublished results. Similar methodology has been used by others to identify new members of the herpesvirus family (VanDevanter *et al.,* 1996;Rose *et al.,* 1997;Ehlers *et al.,* 1999;Rivadeneira *et al.,* 1999;Chmielewicz *et al.,* 2001).

Materials and Methods

Sample selection and experimental approach: Two series of HD cases were investigated for the presence of herpesvirus genomes using a degenerate PCR assay based on conserved regions of the herpesvirus polymerase protein. The first series, which comprised 28 non-selected biopsy samples from a single institution, was used to determine whether the assay was able to detect EBV in EBV+HD cases and thereby validate this experimental approach. At the time of analysis the EBV status of these biopsies was not known but was subsequently determined by EBV EBER *in situ* hybridisation (see below). Samples from the first series were investigated using a single round of PCR. A second series of 17 pre-selected biopsies was then investigated in order to determine whether hitherto unknown herpesvirus genomes are detectable in non-EBV-associated cases. Selection criteria were: classical HD following histological review; non-EBV-associated on the basis of EBV EBER *in situ* hybridisation; sufficient available DNA (Table 1). In order to decrease the likelihood of missing a herpesvirus genome with incomplete homology to our primers, the assay was used in a semi-nested format in the latter analysis; modification of the primers prior to this analysis also resulted in an increase in assay sensitivity. The second case series was also screened for known HHVs using specific quantitative PCR (qPCR) assays.

EBV status: In order to determine the EBV status of Hodgkin's tumours, EBV EBER *in situ* hybridisation analysis was performed on sections of routinely fixed, paraffin-embedded material using a commercially available kit (Vector Laboratories, Peterborough, UK). Cases were classified as EBV-associated or EBV+HD if Reed-Sternberg cells were positive in this assay. The approximate number of EBV-positive bystander cells in sections from non-EBV-associated cases was also recorded.

DNA extraction: DNA was extracted from fresh or frozen biopsy samples that had been mechanically disrupted using a MediMachine (DAKO, High Wycombe, UK).

Proteinase K digestion was followed by extraction with organic solvents and ethanol precipitation as previously described (Trainor *et al.,* 1982).

Degenerate PCR assay for herpesviruses: All the available amino acid sequences of herpesvirus polymerases, with the exception of those from fish viruses, were aligned and regions of homology identified (Figure 1). Primers were designed to include all possible coding sequences of three well-conserved pentapeptides (Figure 1). Clamp sequences were then added to the 5' ends of the degenerate sequences; initially clamps consisted of non-viral sequences with a specified T_m but in the modified primers (primers 1A, 1B and 2) used in the analysis of the second case series clamps were derived from the consensus nucleotide sequence of HHV genomes in this region. In order to reduce the degeneracy of primer pools, and thereby increase sensitivity, the synthesis of the 5' outer primer was split into two (1A and 1B) based on differential codon usage of serine. Likewise the synthesis of the inner primer was split into two, with primers 3A and 3B having codons for phenylalanine and valine respectively. Samples were assayed with both outer primers sets and a semi-nested analysis was performed in the investigation of the second case series. Six separate PCRs were therefore performed in the analysis of the latter cases and these have been designated PCRs 1-6 (Figure 1, Table 1).

Extensive optimisation was performed in order to ensure that the assay had sufficient sensitivity to detect a single herpesvirus genome present in <1% of the cells in a sample. Reactions included 1 µg of template DNA, 1.8 units of AmpliTaq thermostable polymerase, 2 mM $MgCl_2$ and buffer II (all from Applied Biosystems, Warrington, UK), 200 µM nucleotides (Amersham Pharmacia Biotech, Buckinghamshire, UK), TaqStart antibody (Clontech, Cambridge, UK), and 10% glycerol or Q Solution (Qiagen, Crawley, UK). 5' primers were labelled with the fluorochrome Fam (Cruachem, Glasgow, UK) and all primers were used at an optimised concentration of 4 µM. PCRs were performed in a total volume of 50 µl and thermal cycling was performed on a RoboCycler Gradient 96 thermal cycler

(Stratagene, Cambridge, UK) or, in the second series of experiments, a GeneAmp PCR System 2400 (Applied Biosystems). Cycling conditions were: 94°C for 5 min, followed by five cycles of 94°C for 1 min; 44°C for 2 min; 72°C for 3 min, followed by 30 (series1) or 35 cycles (series 2) of 94°C for 1 min; 55°C for 2 min; 72°C for 3 min followed by a final extension step at 72°C for 7 min. In semi-nested reactions, 1 µl of first round product was used as template. Amplification products from both first round and nested reactions were analysed on an ABI PRISM™ 310 or 3100 Genetic Analyzer using GeneScan™ Analysis Software (Applied Biosystems). This gave greater sensitivity than conventional gel analysis, and improved resolution of fragments allowed better discrimination of non-specific and specific amplification products.

Positive controls consisted of DNA from the EBV-positive cell line Raji; HHV-6-infected JJhan cells; and a HCMV-infected tissue culture supernatant. Negative controls containing water were included after every two samples and placental DNA was used to control for non-specificity.

Nucleotide sequence analysis: Sequence analysis was performed to determine the identity of, or further characterise, all PCR products in the anticipated size range for herpesvirus products (236 +/- 50 bases). PCR products were gel purified and cloned into pCR2.1 using a TA cloning kit (Invitrogen, Groningen, The Netherlands). At least 6 clones derived from each 'positive' PCR product were sequenced using universal primers and the Big Dye™ Terminator v2.0 cycle sequencing kit (Applied Biosystems), according to the manufacturer's instructions. Thermal cycling was performed on a GeneAmp PCR System 2400 (Applied Biosystems) and unincorporated fluorescent nucleotides were removed using gel filtration (Edge Biosystems Performa DTR gel filtration kits, Vh Bio, Newcastle-upon-Tyne, UK). Products were analysed on an ABI PRISM™ 310 or 3100 Genetic Analyzer (Applied Biosystems).

Herpesvirus-specific PCR analysis: The second series of cases was screened with qPCR assays for all known HHVs, with the exception of herpes simplex virus (HSV) 2, in order to quantify the levels of viral genomes present and to aid characterisation of fragments amplified in the degenerate assay. TaqMan® methodology (Applied Biosystems) was applied and assays were designed using Primer Express™ version 1.0 software (Applied Biosystems); primers and probe sequences are shown in Table 2. Reactions included 100 ng of template DNA, primers at 50 nM (EBV, HHV-6 variants A and B, HHV-8 and β-globin) or 300 nM, probes at 200 nM, and TaqMan® Universal PCR Mastermix (Applied Biosytems) in a total volume of 50 µl. Thermal cycling and analysis were performed on an ABI PRISM™ 7700 Sequence Detection System (Applied Biosystems) using the default thermal cycling parameters. Positive controls included DNA from: clinical samples infected with HSV1 and VZV; the Raji cell line; a HCMV-infected tissue culture supernatant; JJhan cells infected with HHV-6; SupT1 cells infected with HHV-7; and the BCP-1 cell line (HHV-8). Negative controls containing water in place of DNA template were analysed after every two samples. A β-globin assay was used to quantify the amount of amplifiable DNA within samples as previously described (Gallagher *et al.,* 1999).

Results

Analysis of the first series of non-selected HD cases, using the outer primers 1A and 2 (PCR 1), revealed five samples with clear peaks in the expected size range on the electropherogram tracings (Figure 1c). A further three samples gave rise to small peaks in the same size range. Sequence analysis revealed that all of the corresponding DNA fragments were derived from EBV. EBER *in situ* hybridisation showed that the five samples with clear positive results were from the only EBV-associated cases in this series; EBV was detected in bystander cells in several other cases including the three with weak positive results. The EBV-association

rate (5/28) in this series was unexpectedly low for non-selected UK samples and we can only attribute this to the small sample size. Nevertheless, the results clearly demonstrate that this PCR assay can reliably and consistently detect EBV genomes present in Reed-Sternberg cells, thus validating this approach for the detection of novel herpesviruses in HD.

Following the analysis of the first case series, the primer clamp sequences and assay conditions were modified slightly, see Materials and Methods, to optimise sensitivity. In modified format, the semi-nested assay was able to reliably detect <100 EBV and HHV-6 genomes and <1000 HHV-7 genomes in a background of 1 μg DNA (data not shown). In order to look for currently unknown herpesvirus genomes, samples from 17 cases of non-EBV-associated, classical HD were tested using the semi-nested assay (Figure 1, Table 1). One sample was weakly positive using the outer primers (1A and 2), however, the majority of samples scored 'positive' in at least one of the nested assays (Table 1, Figure 1d). Extensive sequence analysis of these products was carried out, but in all cases fragments corresponded to known HHVs. EBV was the most frequently detected virus, followed by HHV-6 variant B and HHV-7. As anticipated, there was cross-reactivity of primer sets (Table 1).

Specific PCR assays for known HHVs were performed on samples from the second case series. Samples from 12 cases were positive for one or more herpesvirus: EBV (n=5), HHV-6 variant B (n=3) or HHV-7 (n=9). Genome copy numbers were extrapolated from standard curves and normalised using the results of the β-globin qPCR assay; since it is difficult to determine precise copy numbers, results have been categorised into groups containing: no; 1-9; 10-49; 50-99; and 100-350 herpesvirus genomes/100 ng amplifiable DNA (Table 1). One sample (case 3) contained a moderate number of HHV-6 variant B genomes (equivalent to ~1 copy per 45 cells) whereas all other samples contained only a low level of herpesvirus genomes. Detection of EBV in PCR assays correlated with detection

of EBER RNAs in bystander cells by *in situ* hybridisation. No samples were positive for HSV-1, VZV, HCMV, HHV-6 variant A or HHV-8.

In general there was a good correlation between the results of the qPCR and degenerate assays. In 6 samples, low level HHV-7 infection was detected by qPCR but not by the degenerate PCR; this result was consistent with the known sensitivity of the degenerate assay. In 2 samples the degenerate assay failed to detect EBV genomes that were detected at low copy number by qPCR. The degenerate assay picked up EBV in 4 samples and HHV-6 variant B in 3 samples that were negative for these viruses by qPCR. This most probably reflects the fact that 10-fold more DNA was used in the degenerate assays than in the qPCR, and also that the degenerate assay detects EBV and HHV-6 with high sensitivity. In two of the latter cases EBV was detected using PCR 5; although this primer combination was designed for identification of HHV-6, -7 and related viruses, we have previously observed that these primers efficiently amplify EBV.

Discussion

The aim of our study was to identify novel herpesvirus genomes in non-EBV-associated HD using a degenerate PCR strategy. Following validation of the experimental approach, a semi-nested PCR assay with the potential to detect all herpesvirus genomes of mammals, birds, amphibians and reptiles was used to investigate a series of biopsy samples. Herpesvirus *pol* sequences were amplified from the majority of samples tested, however, sequence analysis revealed that all were derived from known lymphotropic herpesviruses, namely EBV, HHV-6 and HHV-7. No new viruses were detected.

Quantitative PCR was used in parallel to detect and quantify the levels of known HHVs within biopsies. Using this methodology genomes of one or more herpesvirus were detected in most samples; HHV-7 was the most frequently

detected herpesvirus followed by EBV and HHV-6 variant B. The level of infection by these viruses was low and genome copy numbers were not consistent with infection of all Reed-Sternberg cells within lesions. For samples that were EBV-positive by PCR, EBER *in situ* hybridisation demonstrated that the virus was present in bystander cells and not Reed-Sternberg cells. There was no evidence to suggest direct involvement of any of these viruses in the pathogenesis of non-EBV-associated HD.

Comparison of the results of degenerate and specific PCR assays, coupled with formal assessments of assay sensitivity, underscores the sensitivity of this semi-nested degenerate PCR approach. The assay clearly has sufficient sensitivity to detect infection of Reed-Sternberg cells in HD. In addition, we have demonstrated that the assay is able to detect a wide range of herpesvirus genomes in clinical samples (this study and unpublished data). Although it is not possible to definitively exclude the presence of an unknown herpesvirus, our study suggests that it is highly unlikely that another herpesvirus is *directly* involved in the pathogenesis of HD.

The epidemiological features of young adult HD, coupled with the age distribution of EBV-negative HD cases, suggests that an infectious agent is involved in disease pathogenesis. A 'hit-and-run' mechanism involving EBV has been suggested (Ambinder 2000), however, some patients with HD have never been infected with EBV and so this mechanism cannot account for all cases,(Chapman *et al.,* 2001) and unpublished results. Future studies should therefore focus on other virus families and indirect involvement of infectious agents.

Acknowledgements

We are indebted to the many pathologists who contributed samples to this study, in particular Nigel Kirkham. This work was supported by a LRF specialist programme grant.

Table 1. Patient details and PCR results

Case	Sex	Age	Diagnosis	EBV	HHV-6B	HHV-7	PCR 1	PCR 2	PCR 3	PCR 4	PCR 5	PCR 6
1	M	42	HDMC	-	-	<50	-	-	-	-	HHV-7	-
2	F	24	HDNS	-	-	-	-	-	-	-	EBV	-
3	F	16	HDNS	<100	<350	<10	+/-	-	EBV	EBV	HHV-6B EBV	-
4	F	19	HDMC	-	-	-	-	-	EBV	-	-	-
5	M	19	HDMC	<10	-	-	-	-	-	EBV	-	-
6	F	25	HDNS	-	-	<50	-	-	EBV	-	HHV-6B HHV-7	-
7	M	20	HDNS	-	-	-	-	-	-	-	-	-
8	F	36	HDNS	-	-	-	-	-	-	-	HHV-6B	-
9	M	43	HDMC	<50	-	<50	-	-	-	-	-	-
10	M	38	HDNS	-	-	<50	-	-	-	-	HHV-6B EBV	-
11	F	41	HDNS	<10	-	-	-	-	-	-	-	-
12	F	54	cHD	<10	-	<10	-	-	EBV	EBV	EBV	-
13	M	19	HDNS	-	<10	<50	-	-	EBV	-	HHV-6B	-
14	M	31	HDNS	-	-	-	-	-	-	-	-	-
15	F	15	HDNS	-	-	<50	-	-	-	-	-	-
16	F	46	HDNS	-	-	<10	-	-	-	-	HHV-7	-
17	M	27	HDNS	-	<10	-	-	-	-	-	HHV-6B	-

M, male; F, female; HDMC, mixed cellularity Hodgkin's disease; HDNS, nodular sclerosis Hodgkin's disease; cHD, classical Hodgkin's disease, further subtyping not possible. Positive results in quantitative PCR assays are shown. Copy numbers of viral genomes were extrapolated from standard curves and normalised using quantitative PCR results for β-globin. Results are grouped according to the number of viral genomes per 100 ng of amplifiable DNA: -, negative; <10; 1-9 genomes; <50; 10-49 genomes; <100; 50-99 genomes; <350, 100-349 genomes. The results of the degenerate PCR assays are tabulated according to the primer set used (Figure 1). Amplification products were analysed using GeneScan™ Analysis Software. Sequence analysis was performed whenever a fragment of the appropriate size was identified and the identity of the corresponding viral genome(s) is shown; -, indicates that no fragments in the expected size range were detected. Since primers 1A and 1B and also 3A and 3B are very similar, cross-reactivity is apparent particularly when larger numbers of viral genomes are present.

Table 2. Degenerate primers

Degenerate primers based on herpesvirus polymerase sequences[1]	
Outer primers:	
5' Primer 1A	*GACTTTCCAAGTTTC*TAYCCNAGYATHAT
5' Primer 1B	*GACTTTCCAAGTTTC*TAYCCNTCNATHAT
3' Primer 2	*ACAAACATACAGTCC*GTRTCNCCRTADAT
Inner primers:	
5' Primer 3A	*GTTTGATGCCGACCT*TAYGGNTTYACNGG
5' Primer 3B	*GTTTGATGCCGACCT*TAYGGNGTNACNGG
Specific assays[2]	
HSV	
5' primer	AGCAGGCCGCCATCAA
Probe	CCGTGAACCCGTACACCGAGTTACACA
3' primer	CAGGAGTCCGTGCTGCACT
VZV	
5' primer	ACTGGAGTTGCGCAGGGA
Probe	TTCTGCCATGTTTATACGTAGCGGCCA
3' primer	TGACGGCCAATTGTAGTGACA
EBV	
5' primer	AGTCCTTCTTGGCTAGTCTGTTGAC
Probe	CATCAAGAAGCTGCTGGCGGCC
3' primer	CTTTGGCGCGGATCCTC
HCMV	
5' primer	TGCGCGAGTGTCAAGACC
Probe	TCGAGCAGCATACGGCGCACA
3' primer	TTGAGCGCCATCTGTTCCTT
HHV-6A	
5' primer	GGATGAGACTCATCGGTTTGTG
Probe	TCCCAAGCACAGACTCACGGATACAAGG
3' primer	GGCCAGCCAGTCCTTTAGTAGA
HHV-6B	
5' primer	GGATGAGACCCATCGGTTTGTG
Probe	TTCCAAGCACAGACTCGCGAACACAAGG
3' primer	GGCCAGCCAGTCCTTTAGTAGA
HHV-7	
5' primer	CGCCATGTATTCGGACACTTT
Probe	CACCGCGTGTCAATCCAAATTTCTCAAT
3' primer	CCTCTATTCCAAATGTCCCTGAA
HHV-8	
5' primer	GCGAAAGATGCTGGAGAGATCT
Probe	CCGGCGAGATGGCCTCTACAAAGG
3' primer	AGGAGACCCGCTAGGCGT
β-globin	
5' primer	GGCAACCCTAAGGTGAAGGC
Probe	CATGGCAAGAAAGTGCTCGGTGCCT
3' primer	GGTGAGCCAGGCCATCACTA

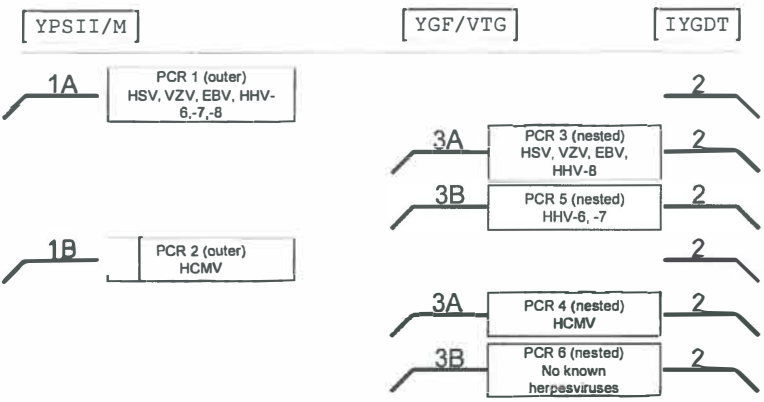
[1]**Degenerate primers**. Primers were designed to include 14 nucleotides corresponding to a conserved pentapeptide motif. A further 15 nucleotides, shown in italics, were added to the 5' end of the primers to serve as a clamp and increase the overall T_m of the primers. The clamp sequences of the outer primers were derived from the consensus nucleotide sequence of the human herpesvirus sequences in this region. N=A+G+C+T; Y=C+T; H+A+C+T; R+A+G and D=A+G+T. [2]**Specific primers:** The primers used in the virus-specific assays are all within the sequence amplified by the outer set of degenerate primers. HHV-6A and B, HHV-6 variants A and B respectively. Primer sets for EBV, HHV-6A, -7, -8 and β-globin have been described previously (Gallagher *et al.,* 1999;MacKenzie *et al.,* 2001).

Figure 1. Degenerate PCR strategy for the detection of herpesvirus genomes and representative results. Panel a) The amino acid sequence of the polymerase proteins of all herpesviruses, where available, were aligned and conserved sequences identified. The sequence of three well-conserved pentapeptides is shown in single letter amino acid code – these sequences are conserved across all herpesviruses, with the exception of fish viruses, although only the human herpesvirus sequences are shown. HSV, herpes simplex virus; VZV, Varicella zoster virus; EBV, Epstein-Barr virus; HCMV, human cytomegalovirus; HHV, human herpesvirus. Panel b) Degenerate PCR primers containing 14 nucleotides corresponding to all possible codon usages of the relevant pentapeptides and a 5' clamp sequence were synthesised and used in the combinations indicated. The clamp sequences of the outer primers (1A, 1B and 2) were based on the consensus sequence of the known human herpesviruses in this region. To reduce degeneracy and increase sensitivity, the synthesis of primer 1 was split into two according to the codon usage for serine. The substitution of methionine for isoleucine in the N-terminal peptide does not affect primer design since the first two bases of the respective codons are identical. Primer 3 was split into two syntheses with primers 3A and 3B having codons for phenylalanine and valine, respectively. PCRs that will preferentially amplify the known human herpesviruses are indicated. Panel c) Representative electropherogram showing products from a sample of EBV-associated HD amplified with primers 1A and 2 (PCR 1). A clear peak (shaded) is visible in the anticipated size range for herpesvirus fragments (533 base pairs) and sequence analysis confirmed that this was EBV. Size markers are indicated with an asterisk. Panel d) Electropherogram showing amplification products from case 17, amplified using PCR 5. A peak is visible (shaded) in the expected size range (236 base pairs) and was subsequently shown to represent HHV-6 variant B. Size markers are indicated with an asterisk.

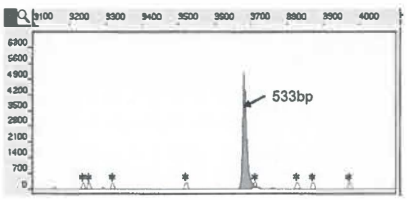
a

HSV1	YPSII	YGFTG	IYGDT
HSV2	-----	-----	-----
VZV	-----	-----	-----
EBV	-----	-----	-----
HCMV	-----	-----	-----
HHV-6	----M	--V--	-----
HHV-7	----M	--V--	-----
HHV-8	-----	-----	-----

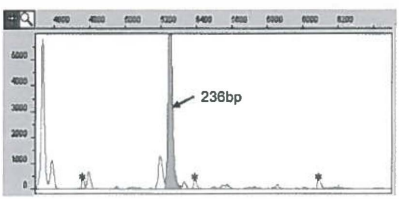
b



c



d



Chapter 5

Viruses and Hodgkin lymphoma: no evidence of polyomavirus genomes in tumour biopsies

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Leuk lymphoma 2006 July; 47(7):1315-21

Abstract

The epidemiology of young adult Hodgkin lymphoma (HL) suggests that delayed exposure to a common childhood pathogen may be involved in disease pathogenesis. The Epstein-Barr virus (EBV) is associated with a proportion of cases but cases of young adult HL in westernised countries are less frequently EBV-associated than cases in other age groups and geographical locales. This study investigated the possibility that polyomaviruses might be involved in the aetiology of HL by analysing a series of 35 cases of classical HL using both specific and degenerate PCR assays for polyomavirus genomes. No positive results were obtained, indicating that it is highly unlikely that this virus family is directly involved in the pathogenesis of HL.

Introduction

The epidemiological features of Hodgkin lymphoma (HL) suggest that an infectious agent may be involved in the pathogenesis of this unusual malignancy (Gutensohn and Cole 1980). The herpesvirus Epstein-Barr virus (EBV) is associated with a proportion of cases and this association is believed to be causal (IARC 1997;Jarrett 2002). In western countries around one third of all cases are EBV-associated, but this proportion varies by geographical locale, age and sex (Jarrett 2002;Jarrett *et al.,* 2003). We have suggested a four disease model of HL which divides HL into four subgroups on the basis of age, EBV-association and age of exposure to EBV (Jarrett 2002). The model includes three EBV-associated subgroups of cases: a childhood subgroup accounting for most cases occurring in early childhood; a young adult subgroup associated with delayed exposure to EBV; and an older adult subgroup, which we speculate is associated with dysregulation of the normal equilibrium between host immunity and EBV. The fourth subgroup is non-EBV associated and accounts for the majority of cases of HL in western countries. It has a unimodal age incidence curve with a peak incidence between the age of 15-34 years. Most young adult HL cases are included in this group and it is therefore likely that the risk factors previously described for young adult cases in developed countries apply to this EBV-negative subgroup of cases.

Risk factors indicative of a high standard of living in early childhood consistently show an association with increased risk of developing young adult HL (Gutensohn and Cole 1980;Gutensohn 1982;Gutensohn and Shapiro 1982;Glaser 1987;Alexander *et al.,* 1991a). It has been inferred from these data that delayed exposure to a common infectious agent is involved in disease pathogenesis. In support of this, we and others have found that young adults with HL report fewer common childhood infections than controls (Paffenbarger *et al.,* 1977;Alexander *et al.,* 2000). Societal changes occurring over recent decades are likely to have influenced patterns of childhood infections but, consistent with the above hypothesis, a recent study found that attendance at nursery school or day care was

associated with a decreased risk of HL (Chang *et al.,* 2004). These data suggest that any virus associated with HL is likely to be widespread and infect many or most individuals at a young age. Herpesviruses, including EBV, fulfil these criteria; however, a previous study from our group using a degenerate PCR assay to detect herpesvirus genomes found no evidence for the presence of herpesviruses, other than EBV, at a significant level in HL biopsies (Gallagher *et al.,* 2002).

Polyomaviruses are also widely distributed in nature, infect most individuals at a young age and are known to be oncogenic in experimental systems. JC virus (JCV) and BK virus (BKV), the two known human polyomaviruses, both establish persistent infections and in immunocompromised persons are associated with progressive multifocal leukoencephalopathy and urinary tract pathology, respectively (Cole and Conzen 2001). Both viruses have been associated with malignant disease but these associations are controversial. SV40 is a related primate virus that does not naturally infect humans but may have been introduced into human populations as a result of contamination of polio vaccines in the 1950s and early 1960s (Shah and Nathanson 1976). SV40 genomes have been reported to be detectable in a high proportion of non-Hodgkin's lymphoma samples in the US (Vilchez *et al.,* 2002;Shivapurkar *et al.,* 2002), but we and others have not detected this virus in samples from the UK, France and Canada (MacKenzie *et al.,* 2003;Brousset *et al.,* 2004). Other investigators have detected SV40 genomes in an intermediate proportion of lymphoma cases (Martini *et al.,* 1998;Nakatsuka *et al.,* 2003). The reasons for these different findings are not clear; geographical differences in the administration of infected polio vaccines are unlikely and therefore technical factors such as differences in assay sensitivity (McNees *et al.,* 2005) and the contamination of DNA samples with SV40, particularly the large T sequences frequently found in plasmid vectors (Lopez-Rios *et al.,* 2004), would seem to be more plausible explanations.

Volter *et al* (1997) previously investigated five cases of HL for the presence of polyomaviruses using degenerate PCR assays based on well conserved amino

acid motifs within the structural protein VP1 (Volter *et al.,* 1997). Negative results were obtained but the number of cases examined was small, HL case details were not given and the reported assay sensitivities were not sufficient to detect single copy genomes present in Hodgkin and Reed-Sternberg (HRS) cells, the tumour cells in HL.

The aim of the present study was to determine whether polyomavirus genomes are present within HL tumours. A series of 35 HL cases was examined using specific assays for JCV, BKV and SV40 and also degenerate PCR assays, which have the potential to detect novel polyomaviruses. Degenerate assays were based on T antigen sequences since defective, integrated genomes lacking T antigen coding sequences are unlikely to be associated with transformation (Cole and Conzen 2001). We avoided sequences in the 5' end of the large T gene as SV40 sequences from this region are present in many cloning vectors and are a potential source of PCR contamination (Lopez-Rios *et al.,* 2004). Since the tumour cells in HL, the HRS cells, generally constitute 1% or less of the total cellular mass, we aimed to develop assays with the ability to detect a single copy genome present in <1% of the cells in any sample. In addition, the study was limited to cases of classical HL (cHL); nodular lymphocyte predominant HL was excluded as this histological subtype is thought to represent a distinct entity with a different cell of origin (Stein 2001).

Materials and Methods

Clinical Samples: Lymph node biopsy samples from 35 non-selected cases of cHL were investigated (Table I); 17 cases were part of a previous study examining the association between SV40 and lymphoma (MacKenzie *et al.,* 2003). Flow cytometric analysis was performed on 29 of these cases in order to determine the approximate number of HRS cells in the biopsy samples. Briefly, single cell suspensions were incubated with a panel of antibodies including BerH2 (Dako,

Cambridgeshire, UK), which is reactive with CD30, and results analysed on an EPICS Elite (Beckman Coulter, High Wycombe, UK). The overall percentage of CD30-positive cells was recorded using the number of viable cells as the denominator. DNA was extracted from fresh or frozen lymph nodes using either proteinase K digestion followed by extraction with organic solvents, or the QIAamp® DNA Blood Mini kit (Qiagen, Crawley, UK). The EBV status of tumours was determined using EBV EBER *in situ* hybridisation. Briefly, sections of paraffin-embedded lymph node biopsies were hybridised with a commercially available probe (Vector Laboratories, Peterborough, UK) and reactivity detected using a hybridisation kit (Dako). Cases in which the HRS cells were positive are designated EBV-associated or EBV-positive. This study was approved by a multicentre research ethics committee and samples were assayed in an anonymised fashion.

Quantitative Real-Time PCR analysis: DNA samples (500 ng) were screened using quantitative PCR assays for JCV, BKV and SV40, as previously described (MacKenzie *et al.,* 2003). We have previously shown that the SV40 assay can consistently detect 10 copies of SV40 DNA in a background of 1 µg high molecular weight DNA (MacKenzie *et al.,* 2003). To determine the sensitivity of the BK and JC assays, multiple replicates of dilutions of plasmids carrying the complete JCV and BKV genomes were spiked into 1 µg of DNA from the J-JHAN cell line. A quantitative PCR assay for β-globin was also performed to confirm that all samples contained sufficient amplifiable DNA (Gallagher *et al.,* 1999). Thermal cycling and analysis were performed on an ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Warrington, UK).

Degenerate PCR for Polyomavirus Genomes

Assay design: Amino acid sequences from all available large T antigen sequences were aligned using ClustalW, courtesy of the BCM search launcher (**http://dot.imgen.bcm.tmc.edu/multi-align/multi-align.html**), and conserved

blocks of amino acids were identified (Figure 1). Three peptide motifs were selected for design of degenerate primers. The single forward primer was derived from the sequence E/DDVKG, which is conserved in all polyomaviruses except the Kilham strain of murine polyomavirus. Since codons for E (glutamic acid) and D (aspartic acid) have the same nucleotides in positions 1 and 2, both amino acids are covered by this primer (Table 2). The conserved sequence VNLE was selected for design of the reverse primer; two primers were synthesised with one including all nucleotide sequences encoding KVNLE and the other including all those encoding PVNLE (Table 2). A third reverse primer, based on the sequence TMNEY, was also synthesised (Table 2); although this sequence is not perfectly conserved across all polyomaviruses, the resultant primer has low degeneracy (16-fold) and, therefore, use of this primer was predicted to result in an assay with superior sensitivity. Clamp sequences of 15-16 nucleotides were added to the 5' end of each primer. These sequences were based on the consensus nucleotide sequence at the relevant position of the viral genomes, with some modifications to ensure a reasonably high melting temperature (T_m). Primers including clamp sequences plus two nucleotides of non-degenerate polyomavirus sequence were also synthesised separately for use in second round PCRs (Table 2). The EDVKG and CLAMP-EDVKG primers were labelled with FAM and the CLAMP-VNLE primer was labelled with HEX; all primers were synthesised by TAG Newcastle Ltd (Gateshead, UK).

Assay specificity and sensitivity: The following templates were used to validate the specificity of the assay: SV40 viral DNA (Form 1, Invitrogen, Paisley, UK); DNA from the COS-7 cell line, which contains integrated SV40 (Gluzman 1981); DNA extracted from cell culture supernatants containing bovine polyomavirus (BPyV); and plasmids containing the complete JCV, BKV, lymphotropic papovavirus (LPV) and hamster polyomavirus (HaPV) genomes. All samples were assayed in a background of 1 µg of DNA from the J-JHAN cell line. In addition, DNA from the rabbit papovavirus (strain 443; LGC Promochem, London UK), a known but

uncharacterised polyomavirus, was tested. DNA was extracted from tissue culture supernatants using the QIAamp® UltraSens® Virus Kit (Qiagen).

Replicates of serial 10-fold dilutions, predicted to contain from 10^6 to 10 viral genomes, of SV40 and BPyV were assayed in a background of high molecular weight DNA to determine assay sensitivity. Semi-log dilutions of viral templates were used to determine sensitivities in the range $10^4 - 10^2$ viral genomes per µg DNA.

Hodgkin lymphoma samples: Following optimisation and validation of the degenerate assays, DNA from the HL biopsies was assayed using each primer set. In addition, a second round PCR was performed using the CLAMP primers where appropriate (Table 2). Negative controls, with water replacing the DNA template, were included after each sample in order to control for PCR contamination, and J-JHAN DNA (1 µg) was used to control for non-specific amplification. A conventional PCR assay for β-globin, which amplifies a 110 base pair product, was performed on all samples to verify the presence of amplifiable DNA (Saiki *et al.,* 1988).

PCR reaction mixtures contained 1 µg DNA template, 4 µM of each primer, 200 µM dNTPs (Amersham Pharmacia Biotech, Buckinghamshire, UK), 10x PCR buffer containing 1.5 mM $MgCl_2$, 5x Q-solution and 1.25 U HotStarTaq DNA polymerase (all from Qiagen). Thermal cycling was performed on a GeneAmp PCR System 2400 (Applied Biosystems) using the following parameters: 95°C for 15 minutes, followed by five cycles of 94°C for 60 s; 44°C for 2 minutes; 72°C for 3 minutes, followed by 35 cycles of 94°C for 60 s; 55°C for 2 minutes; 72°C for 3 minutes followed by a final extension step at 72°C for 7 minutes. Following amplification with primer pair EDVKG/KVNLE or EDVKG/PVNLE, 1 µl of first round products was subjected to a further 20 cycles of amplification using primers CLAMP-EDVKG and CLAMP-VNLE and an annealing temperature of 45°C. Reaction products were analysed on an ABI PRISM® 310 Genetic Analyzer using GeneScan analysis

(Applied Biosystems) as this method gives greater sensitivity and fragment size resolution than conventional electrophoresis.

Nucleotide sequence analysis: Sequence analysis was performed to further characterise degenerate PCR products in the anticipated size range for amplicons of polyomavirus genomes. Amplification products were cloned using the TOPO™ TA Cloning® kit (Invitrogen) and sequencing performed using the Big Dye® Terminators v3.1 Cycle Sequencing Kit and an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). Nucleotide sequences were subjected to blastn searches (http://www.ncbi.nlm.nih.gov/BLAST).

Results

Flow Cytometry: Single cell suspensions from 29 of the 35 cases were analysed by flow cytometry and the results are shown in Table I. In the majority of cases (24/29), ≥1% of the viable cells were reactive with the BerH2 antibody indicating cell surface CD30-positivity; although CD30 staining is not entirely specific to HRS cells, the vast majority of CD30-positive cells within HL tumours would be expected to be HRS cells.

Quantitative Real-Time PCR Analysis: HL samples were screened using sensitive PCR assays for the known human polyomaviruses JC and BK and the primate virus SV40. All samples were negative in these assays despite the presence of amplifiable DNA. Analysis of replicate dilutions of plasmids containing full length JCV and BKV genomes confirmed that all of these assays could consistently detect 10 copies of viral DNA

Degenerate PCR for Polyomavirus Genomes

Specificity and sensitivity: Degenerate PCR assays based on the large T antigen of polyomaviruses were designed and validated. Primers were derived from the 3' region of the large T gene and therefore will not amplify the SV40 sequences that are most frequently present in cloning vectors (Lopez-Rios *et al.,* 2004). Primer pairs had the expected specificities: JCV, BKV, SV40, LPV and HaPV templates were amplified using the reverse primer KVNLE, whereas BPyV was amplified more efficiently using the PVNLE reverse primer (Figure 2a). JCV, BKV and LPV were successfully amplified using the reverse primer TMNEY; despite an imperfect sequence match, SV40 was also efficiently amplified using this primer (Figure 2a). All amplification products were of the expected size – 146 base pairs for the EDVKG/KVNLE or EDVKG/PVNLE assays and 208 base pairs for the EDVKG/TMNEY assay. When DNA from the rabbit papovavirus was used as template, negative results were obtained using the PVNLE and TMNEY reverse primers but a 146 base pair product was generated using the EDVKG/KVNLE primer set (Figure 2b). The nucleotide sequence of this amplification product, and the predicted translation, were found to have homology with known polyomavirus genomes and large T antigens, respectively. The nucleotide sequence has been deposited as GenBank accession number AY911513.

Analysis of serial dilutions of template DNA, in a background of 1 µg of high molecular weight DNA, was performed in order to determine assay sensitivity. To detect a single copy viral genome present in 1% of the cells, the assay should be able to detect 1.5 x 10^3 genomes in a 1 µg DNA sample. Using the EDVKG and KVNLE or PVNLE primers, we were able to detect $10^{3.5}$ genome copies of SV40, JCV, BKV and BPyV, but could not consistently detect 10^3 copies. Using the CLAMP primers in a second round reaction, we were able to detect $10^{2.5}$ SV40 genomes and 10^3 BPyV genomes. As predicted, the assay incorporating the TMNEY primers was most sensitive having the ability to detect $<10^2$ SV40 genomes.

Hodgkin lymphoma samples: DNA samples from 35 cases of cHL were assayed using the above degenerate PCR assays for polyomaviruses, on multiple occasions. No PCR products in the anticipated size range were detected on GeneScan analysis.

Discussion

Sensitive, degenerate PCR assays based on the large T antigen of polyomaviruses were developed and their specificity and sensitivity were validated using a variety of templates. Using this approach, we successfully amplified sequences from the rabbit papovavirus, a known virus for which no sequence information was available, thus confirming the usefulness of these assays for polyomavirus detection.

Both specific and degenerate PCR assays were used to investigate polyomavirus involvement in HL. No positive results were obtained suggesting that it is highly unlikely that a polyomavirus is present within all the HRS cells of HL tumours. The quantitative assays for SV40, JCV and BKV utilise TaqMan® methodology and are therefore very sensitive, having the ability to detect ≤10 copies of the viral genomes in a background of 1 µg high molecular weight DNA. Degenerate PCR assays are inherently less sensitive, but the least sensitive of the semi-nested assays used in this study was able to detect 10^3 genomes in a DNA sample from the equivalent of $1.5x10^5$ cells. Since, in the majority of our samples, >1% of the cells were reactive with CD30 antibodies, this level of sensitivity should be sufficient to detect polyomavirus infection of HRS cells. All of the assays utilised in this analysis were based on large T sequences and it is therefore unlikely that defective, integrated genomes have escaped detection. We cannot exclude the possibility that a small proportion of the total HRS cell population is infected by a polyomavirus or the existence of a hit-and-run mechanism involving polyomaviruses. However, we

believe these results suggest that it is highly unlikely that polyomaviruses are directly involved in the transformation of HRS cells in HL.

The epidemiological features of young adult EBV-negative HL suggest that an infectious agent is involved in disease pathogenesis (Gutensohn and Cole 1980). Previous studies from our laboratory indicate that herpesviruses are unlikely to be directly involved (Gallagher *et al.,* 2002). The inability to detect persistent EBV infection in patients with non-EBV-associated HL using multiple methods; the finding that young adult HL cases with non-EBV-associated disease are more likely to be EBV seronegative than controls; and the lack of defective or rearranged (Gan *et al.,* 2002) EBV genomes in HL biopsies from adult cases, all argue against a 'hit and run' mechanism in cases classified as 'EBV-negative' using *in situ* techniques (Staratschek-Jox *et al.,* 2000;Gallagher *et al.,* 2003;Knecht and Odermatt 2003). This study further suggests that polyomaviruses are not directly implicated. The putative virus remains elusive but complementary techniques for virus discovery, which do not require any *a priori* knowledge of the likely agent, need to be explored before a direct role for viruses in this disease can be excluded.

Acknowledgements

We would like to thank Jacqui Perry, Linda Scobie, David Onions and many collaborating pathologists for their contributions to this study. This work was supported by a Leukaemia Research Fund specialist programme grant.

Table 1. Patient details

Case number	Sex	Age* at diagnosis	Histological subtype	EBV status	%CD30# positive
1	F	24	NSHL	Positive	1
2	M	42	MCHL	Negative	ND
3	M	21	NSHL	Negative	ND
4	M	63	NSHL	Negative	ND
5	F	16	NSHL	Negative	ND
6	F	25	NSHL	Negative	2
7	M	36	NSHL	Positive	3
8	M	29	NSHL	Positive	2
9	M	43	MCHL	Negative	ND
10	M	42	NSHL	Positive	3
11	M	38	NSHL	Negative	3
12	F	41	NSHL	Negative	<1
13	M	19	MCHL	Positive	3
14	M	19	NSHL	Negative	<1
15	F	15	NSHL	Positive	1
16	M	31	NSHL	Negative	2
17	F	33	HL NOS	Positive	<1
18	M	31	NSHL	Positive	<1
19	F	15	NSHL	Negative	2
20	F	46	NSHL	Negative	7
21	M	33	NSHL	Negative	5
22	M	30	NSHL	Positive	ND
23	M	27	NSHL	Negative	8
24	F	76	NSHL	Negative	3
25	F	22	NSHL	Negative	5
26	F	17	NSHL	Negative	4
27	F	14	NSHL	Negative	13
28	M	37	MCHL	Negative	3
29	M	21	NSHL	Positive	6
30	M	27	NSHL	Negative	1
31	F	36	NSHL	Positive	3
32	M	23	MCHL	Positive	1
33	F	22	NSHL	Negative	0
34	F	31	NSHL	Negative	1
35	F	16	NSHL	Negative	1

F, female; M, male; NSHL, nodular sclerosis Hodgkin lymphoma; MCHL, mixed cellularity Hodgkin lymphoma; HL NOS, Hodgkin lymphoma not otherwise specified as further subtyping not possible, ND, not done. *Age in years. #%CD30-positive as defined by flow cytometry using total viable cells as the denominator.

Table 2. Degenerate PCR primers for detection of polyomaviruses

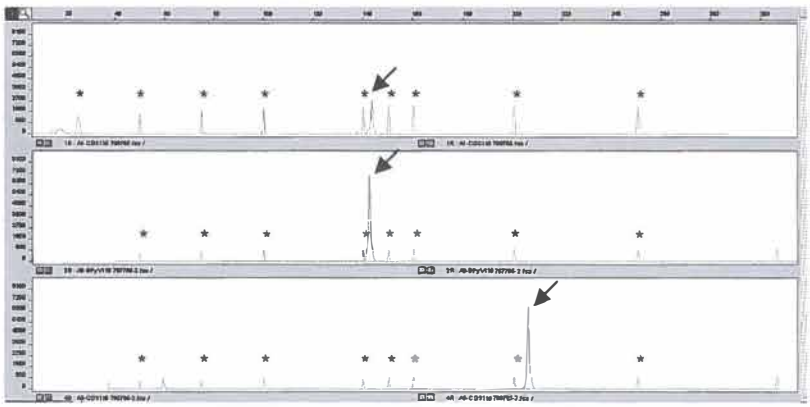
Primers	Fold Degeneracy
Forward Primer EDVKG	
**GTTTATGGTTGTCTTT*GANGAYGTNAARGG	64
Reverse Primer KVNLE	
*GTTTCGGTGTTTCTTT*TCNARRTTNACYTT	128
Reverse Primer PVNLE	
*GTTTCGGTGTTTCTTT*TCNARRTTNACNGG	256
Reverse Primer TMNEY	
*GTTTGAGGCACAGAA*TAYTCRTTCATNGT	16
Forward CLAMP-EDVKG	
*GTTTATGGTTGTCTTTGA	1
Reverse CLAMP-VNLE	
#GTTTCGGTGTTTCTTTTC	1

Clamp sequences are shown in italics. N = A + G + C + T; Y = C + T; and R = A + G. * FAM-labelled oligonucleotides; # HEX-labelled oligonucleotide.

	EDVKG	KVNLE	TMNEY
BKV	-----	-----	-----
JCV	-----	-----	-----
LPV	D----	-----	-----
SV40	-----	-----	----F
HaPV	-----	-----	-A---
Py (Crawford)	-----	-----	-----
Py (strain A2)	-----	-----	-----
Py (strain A3)	-----	-----	-----
Py (Kilham)	-VLAE	-----	-S---
BPyV	-----	P----	-C---
BFDV	-----	P----	---N-
GHPV	-----	P----	---H-

Figure 1. Conserved amino acid motifs within the large T antigen of polyomaviruses
- denotes amino acid identity. BKV, BK virus; JCV, JC virus; LPV, lymphotropic papovavirus; SV40, simian virus 40; HaPV, hamster polyomavirus; Py, murine polyomavirus; BPyV, bovine polyomavirus; BFDV, budgerigar fledgling disease virus; GHPV, goose haemorrhagic polyomavirus.

a



b

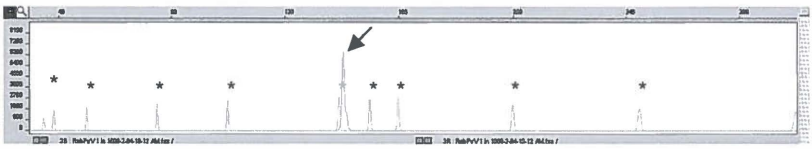


Figure 2. GeneScan analysis of degenerate PCR products. a) Upper panel: amplification of SV40 sequences using primer pair EDKVG and KVNLE. Middle panel: amplification of bovine polyomavirus sequences using primer pair EDKVG and PVNLE. Lower panel: amplification of SV40 sequences using primer pair EDKVG and TMNEY. b) Amplification of partial genomic sequence from rabbit papovavirus. Arrows indicate specific polyomavirus PCR products; *idicates ROX 35O size marker.

Chapter 6

Molecular methods for virus discovery

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Dev Biol (Basel) 2006; 123:77-88

Chapter 6

Abstract

The potential use of novel cell substrates from diverse animal species raises concerns about the transmission of hitherto unknown viral agents. Viruses that do not cause a cytopathic effect in cell culture may escape detection by conventional methods and molecular methods may therefore prove useful for screening for hitherto unknown viruses. This review describes currently used molecular methods for virus discovery, including degenerate PCR assays, representational difference analysis and rolling circle amplification, and summarises the advantages and disadvantages of each technique.

Introduction

Characterizing potential virus contaminants in vaccines is a complex and constantly evolving problem. Contaminants have been associated with the use of primary cell substrates and of non-irradiated materials like trypsin and serum. The move towards using clonally derived cell lines, irradiated serum and recombinant trypsin have been major steps in improving the safety of the new generation of vaccines. At the same time there has been renewed interest in cell substrates, some of them tumourigenic, from species such as dogs, African green monkeys and Syrian hamsters. Of particular concern is that these cells might harbour a virus, which while non-pathogenic in the species of origin, might be tumourigenic in a human host. Virus infections in these cell substrates may not always be revealed by traditional assays that depend on a cytopathic effect, and this has driven the need to implement new technologies for virus detection.

Molecular techniques for virus discovery have been developed over the last ten to fifteen years and now have a proven track record. Early attempts to identify novel viruses by molecular means lacked sensitivity and specificity but modern amplification techniques, such as the polymerase chain reaction (PCR), have revolutionised the field of virus discovery. Currently used methods include degenerate PCR assays, subtractive hybridisation including representational difference analysis (RDA)(Lisitsyn *et al.,* 1993), and the more recently described rolling circle amplification (RCA) technique for detection of closed circular DNA genomes (Rector *et al.,* 2004b). This review will summarise these techniques and their advantages and disadvantages. Additional methodologies, such as screening cDNA expression libraries with immune serum, have been successfully used in the past (Choo *et al.,* 1989) but are less relevant to the analysis of vaccine cell substrates and will not be considered further.

Degenerate PCR assays: Degenerate or redundant PCR assays utilise pools of primers that include most or all of the possible nucleotide sequences encoding a

conserved amino acid motif. They have been used extensively to identify genes encoding proteins related to known proteins, for example new members of protein families or functional homologues in different species. In the context of virus discovery, these assays are used to detect novel viral genomes related to known genomes; they are therefore useful for identification of new members of virus families or subfamilies.

The first step in the design of degenerate assays is to identify proteins that are well conserved among different members of a virus family and to perform an alignment of all the available amino acid sequences. Conserved blocks of sequence are then identified; in our experience conserved pentapeptides are ideal for primer design. A degenerate nucleotide sequence, containing all the possible sequences encoding the pentapeptide, is then derived from the amino acid sequence. The level of degeneracy (i.e., the number of different primers in the primer pool) will depend on the number of codons for each of the amino acids. Since sensitivity is inversely proportional to degeneracy, it is preferable to select conserved peptides that contain amino acids such as methionine, with only one codon, rather than amino acids such as serine, with six; unfortunately, this is not always possible. The degenerate nucleotide sequence corresponding to a pentapeptide is 14 bases long, since the terminal degenerate nucleotide is omitted from the primer. To achieve a reasonable level of sensitivity a primer with a degeneracy of 128-fold or less is desirable.

In order to increase PCR efficiency, a non-degenerate clamp sequence is usually added to the 5' end of the primer; this facilitates annealing of the primer to PCR products generated during the reaction. In our laboratory, the clamp sequence is derived from the consensus **nucleotide** sequence at the relevant position of the viral gene, with some modifications to ensure a reasonably high primer Tm. Since this clamp sequence has homology to the viral target template, it will stabilise primer binding during the early cycles of the PCR. We also usually add the sequence GTTT to the 5' ends of primers to ensure optimal cloning efficiency using

TA cloning methods (Brownstein *et al.,* 1996). The first rounds of thermal cycling are performed using a low annealing temperature (e.g., five cycles with annealing at 44 °C) to allow the degenerate sequence to anneal to the template and then the annealing temperature is raised to facilitate specific amplification of PCR products.

A similar strategy for degenerate PCR design has been independently developed in Tim Rose's laboratory and designated CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primer)(Rose *et al.,* 2003); a software programme is available for primer design at http://blocks.fhcrc.org/codehop.html. The main difference between this method of primer design and that described above is that the clamp sequence is derived from amino acid sequence, using codon preferences based on species.

Degenerate PCR assays are inherently non-specific and lack sensitivity compared to conventional PCR assays. Probes are generally not available, therefore positive results cannot be confirmed by hybridisation; identification of novel viral sequences is therefore dependent on identification of DNA fragments in the anticipated size range and subsequent sequence analysis. For these reasons, we prefer to analyse PCR products using capillary electrophoresis and GeneScan™ analysis (Applied Biosystems, Warrington, UK). This gives improved sensitivity (up to 100-fold) compared to conventional agarose or polyacrylamide gel electrophoresis, and the superior fragment size resolution helps distinguish non-specific amplification products, present in control samples, from fragments of interest (Gallagher *et al.,* 2002).

The major focus of research in our laboratory is the search for a novel viral agent in Hodgkin lymphoma (Jarrett 2002). This malignancy presents many challenges as the malignant cells are fragile, difficult to purify and scarce, usually representing less than 1% of the total cells in the tumour. In order to detect viral genomes present in the malignant cells our degenerate PCR assays have to be able to detect a single copy genome present in 1% of the sample. Since 1 µg of tumour

DNA is generally used as template in our assays, we aim to detect $1.5x10^3$ viral genomes within a background of high molecular weight DNA. The epidemiology of Hodgkin lymphoma suggests that the disease may result from delayed infection with a common infectious agent. We have therefore focused our efforts on the search for new members of virus families that are widespread in the human population and infect most individuals at a young age. For these reasons, we have designed degenerate PCR assays for detection of herpesviruses and polyomaviruses, as described below.

PCR assays for detection of herpesvirus genomes: Degenerate PCR assays have been used successfully to identify many new members of the herpesvirus family (Ehlers *et al.,* 1999;Rivadeneira *et al.,* 1999). Assays are generally based on the polymerase and glycoprotein B proteins, as these are well conserved across family members. Alignment of the available polymerase sequences reveals several blocks of amino acid sequence that are well conserved in all herpesviruses, with the exception of the fish viruses (Fig. 1). We have designed an assay based on the YPSII and IYGDT amino acid motifs (Fig. 1); although YPSII is not absolutely conserved, the substitution of the final isoleucine (I) with methionine (M) does not affect the primer design. Since 144 primers are required to cover all possible nucleotide sequences encoding the pentapeptide YPSII, we have split the synthesis of this primer into two in order to maintain sensitivity. An internal primer, based on the YGF/VTG motif, allows the assay to be run in a semi-nested format (Fig. 1); again, the synthesis of this primer has been split into two, with one primer covering the codon usage of phenylalanine (F) and the other valine (V). Clamp sequences have been added to the 5' ends of the primers, essentially as described above (Table 1).

This assay is extremely sensitive and robust (MacKenzie *et al.,* 2001;Gallagher *et al.,* 2002). Using the outer primer sets we can detect 10^3 copies of a herpesvirus genome present in a background of 1 µg DNA and this sensitivity increases to $<10^2$ copies using the semi-nested primers. We have validated the assay using a wide

range of templates (Fig. 1), and have amplified *pol* gene sequences from porcine cytomegalovirus, a known virus for which no sequence was available at the time of analysis. The assay is relatively simple to perform and can therefore be used for screening purposes. Similar assays have been used by other laboratories to identify novel herpesvirus sequences in a wide range of animal species including non-human primates, pigs, and turtles (Quackenbush *et al.,* 1998;Ehlers *et al.,* 1999;Rivadeneira *et al.,* 1999). Novel herpesvirus sequences were not detected in DNA samples from Hodgkin lymphoma biopsies using this approach (Gallagher *et al.,* 2002).

PCR assays for detection of polyomavirus genomes: Polyomaviruses have well conserved amino acid sequences, particularly in the gene encoding the virus capsid protein VP1 (Volter *et al.,* 1998). However, tumours associated with polyomaviruses frequently contain integrated viral genomes which have lost these sequences, although the gene encoding the T antigen is retained. We therefore elected to design an assay based on the large T antigen, which is less well conserved than the structural proteins. Sequences from SV40, particularly those at the 5' end of the large T gene, are frequently included in plasmid vectors and contamination of DNA samples with these vectors can lead to false positive results in PCR assays for SV40 and related viruses (Lopez-Rios *et al.,* 2004). It is therefore prudent to avoid the 5' part of the T antigen gene when designing primers.

Alignment of the available large T antigen sequences did not reveal any conserved pentapeptides although there were several tetrapeptides that were conserved across all viruses, with the exception of the Kilham strain of mouse polyomavirus (Fig. 2). We selected the E/DDVKG motif for design of the 5' primer, since codons for E (glutamatic acid) and D (aspartic acid) only differ at the third position, and the VNLE motif for design of the 3' primer. Synthesis of the latter primer was split into two, with separate primers covering KVNLE and PVNLE (Fig. 2). An additional 3' primer derived from the amino acid sequence TMNEY was also synthesised;

although this sequence is not conserved in all polyomaviruses, the respective primer has only 16-fold degeneracy and was predicted to give greater sensitivity than the VNLE primers. Clamp sequences were added to all primers, as described above.

The assay was validated using a range of templates including the human viruses JC and BK, the non-human primate virus SV40, hamster polyomavirus and bovine polyomavirus (Wilson et al., submitted). Primer sets had the expected specificities. In addition, sequences from a rabbit polyomavirus, a virus for which no sequence information was available, were amplified (Wilson et al., submitted). Using the KVNLE and PVNLE primers we were able to detect $10^3 – 5x10^3$ viral genomes in a background of 1 µg DNA; as predicted, the assay using the TMNEY primer was more sensitive, detecting $<10^3$ viral genomes in a 1 µg sample.

Detection of polyomavirus sequences in human lymphoma: The detection of SV40 in samples of non-Hodgkin lymphoma has become a controversial issue with some groups detecting viral genomes in >40% of cases (Vilchez *et al.,* 2002), while others have failed to detect evidence of the virus (Brousset *et al.,* 2004). We screened DNA samples from 152 cases of non-Hodgkin lymphoma and 35 cases of Hodgkin lymphoma for the presence of SV40, BK virus and JC virus genomes using specific TaqMan™ assays with negative results (MacKenzie *et al.,* 2003). A previously described consensus PCR assay, based on nucleotide sequences conserved between JC, BK and SV40 genomes, was also used to screen 128 of the non-Hodgkin's lymphoma samples and 18 of the Hodgkin lymphoma samples; again only negative results were obtained. The degenerate assays described above were used to investigate 35 cases of Hodgkin lymphoma but we found no evidence of polyomavirus genomes (Wilson et al., submitted). These results suggest that it is unlikely that SV40 is involved in the pathogenesis of commonly occurring non-Hodgkin lymphomas, and polyomaviruses are unlikely to play a direct role in Hodgkin lymphoma.

The above description provides examples of degenerate PCR assays designed to search for novel DNA tumour viruses. However, these assays can be used to detect a wide range of viruses including RNA and DNA viruses, viruses causing lytic and latent infections, and viruses with circular and linear genomes. For example, degenerate PCR was used to successfully identify a new hantavirus associated with an acute respiratory distress syndrome (Nichol *et al.,* 1993). These assays are sensitive, versatile, robust and relatively easy to perform and can therefore be used for screening purposes. The main disadvantage is that they require knowledge of the viral agent that is likely to be involved and can only be used where sufficient sequence information, from related viruses, is available. Assays will be most reliable when primer design is based on alignment of a large number of viral genomes, as is the case with the herpesvirus assays.

Subtractive hybridisation techniques: Subtractive hybridisation is used to isolate differences between two nucleic acid species. Since viral infection constitutes addition of exogenous nucleic acid sequences to a cell, this methodology can be used to detect RNA or DNA viruses. These assays are more difficult and cumbersome to perform than degenerate PCR assays but have the advantage that no *a priori* knowledge of the potential viral agent is required.

Subtractive hybridisation involves two samples of nucleic acid – the **tester** sample, which includes the **target** sequence of interest, and the **driver** sample. The tester and driver samples are hybridised, allowing complementary strands of nucleic acid to anneal, and then various strategies are used to rescue either single-stranded tester molecules or tester:tester hybrids. The driver and tester samples must be as closely matched as possible in order to minimise differences in nucleic acid composition. For subtractive hybridisations using DNA samples, the driver sample should be derived from a non-affected sample from the same individual, whereas in cDNA subtractions the driver should be from the same cell type (at the same stage of differentiation) as the tester sample. Driver samples are always used in excess in the hybridisation reaction.

Subtractive hybridisation works best when nucleic acid complexity is low and the hybridisation reaction approaches completion. Body fluids, such as serum, and cell culture supernatants probably represent the least complex samples for virus hunting. RNA is considerably less complex than genomic DNA; RNA/cDNA subtractions are therefore simpler to perform than DNA subtractions and a wider choice of methodologies is available. Strategies for subtractive hybridisation using genomic DNA samples generally lack sensitivity because the complexity of the DNA prevents the hybridisation from reaching completion. The representational difference analysis (RDA) protocol circumvents this problem by using only a representation, or defined part, of the human genome in each hybridisation reaction (Lisitsyn *et al.,* 1993). Representations are defined by digestion with a restriction enzyme and subsequent size selection. This results in a reduction of complexity of the DNA sample, which varies depending on the enzyme used.

Following the generation of tester and driver representations, adaptor sequences are ligated to the 5' ends of the tester sample. The tester and driver samples are then hybridised and a 'fill-in reaction' is performed. This results in the addition of nucleotides, complementary to the adaptor molecule, to the 3' ends of all molecules that have hybridised to tester molecules. As a result, tester molecules that hybridised to other tester molecules (tester:tester hybrids) will have adaptor sequences, or their complements, on each end and will therefore be amplified exponentially using the adaptor as primer. Molecules that formed driver:driver hybrids will not have adaptor sequences on either end and will not be amplifiable using the adaptor primer, whereas molecules that were part of tester:driver hybrids will have an adaptor sequence at only one end and will be amplified linearly. Similarly, single stranded tester molecules will be amplified linearly. Each hybridisation and 'fill-in' is therefore followed by a PCR reaction, using the adaptor as primer, which results in the selective enrichment of double-stranded tester molecules. Following amplification, the adaptor sequence is changed and further rounds of hybridisation and amplification are performed. The first round of RDA is

largely subtractive but, as a result of the second order kinetics of reassociation, subsequent rounds have an increased kinetic component.

The chance of detecting a viral genome using RDA increases with genome size since there is a greater chance of fragments from larger genomes being present in the representation. We have estimated that there is a 99% probability of detecting the presence of a herpesvirus genome in a single representation, but two independent representations are required to have a 95% probability of detecting an adenovirus genome and three to four are required for detection of a retrovirus genome.

The RDA protocol requires meticulous bench work but, in our experience, the technique can reliably detect viral genomes present at single copy level. By monitoring the procedure using quantitative PCR, we have shown that fragments of the EBV genome can be amplified >1 million-fold using three rounds of RDA. In our hands the technique does not work reliably when the target fragment is present at lower levels and when the starting DNA samples are not of good quality.

The main advantage of RDA is that no *a priori* knowledge of the infectious agent is required. It is also versatile in that it can be used to detect linear and circular genomes and modified protocols can be used for the detection of RNA viruses and viruses present in body fluids. The need for matched samples, multiple representations and experienced lab workers are the main disadvantages. The technique is not ideal for screening samples and a high index of suspicion is required before embarking on RDA. Nevertheless, RDA has a good track record for virus discovery including the identification of human herpesvirus 8 (Chang *et al.,* 1994), the elusive agent in Kaposi's sarcoma, and the circovirus TTV (Nishizawa *et al.,* 1997).

Rolling circle amplification: Rolling circle amplification (RCA) is a technique used to exponentially amplify single or double-stranded circular DNA templates. It is

widely used as a laboratory method for amplifying small circular molecules, such as plasmids, and has only recently been described as a method for virus discovery (Rector *et al.,* 2004b). RCA utilises bacteriophage ϕ29 DNA polymerase, a high-fidelity enzyme with a strong strand-displacing capability, high processivity and proofreading activity. The processivity, coupled with the strand-displacing capability, enables the generation of tandem-repeat concatemers of circular DNA molecules. Elongation of displaced single-stranded products results in exponential amplification of the template DNA. Random hexamers are used to prime the reaction, eliminating the requirement for custom primers. RCA can be therefore be used to amplify complete, circular DNA genomes without any knowledge of viral genomic sequence.

Rector *et al* (2004) first reported the use of the RCA technique in virus discovery. Using a commercially available kit (TempliPhi 100, Amersham Biosciences, Amersham, UK) they were able to selectively amplify complete genomes from human papillomavirus 16 DNA. Assay sensitivity was improved by increasing the concentration of nucleotides in the reaction. Optimised conditions were then used to isolate and sequence a novel variant of bovine papillomavirus type 1 and a novel papillomavirus from a Florida manatee (Rector *et al.,* 2004a;Rector *et al.,* 2004b) We have used this protocol to successfully amplify complete papillomavirus genomes from equine sarcoid tumours (Fig. 3) and complete polyomavirus genomes, including the genome of the rabbit polyomavirus metioned above. The technique is relatively simple to perform and is suitable for screening purposes. The major disadvantages of RCA are first, it can only be used to selectively amplify circular genomes and secondly, assay sensitivity is decreased when genomic DNA samples are used as template, since the background DNA is also amplified, albeit with lesser efficiency, during the multiple displacement amplification reaction.

Conclusions

The last fifteen years have witnessed the dawn of the molecular era of virus discovery. Several complementary methods for the identification of novel viruses have been developed, each with its own pros and cons. Degenerate PCR has the advantage of sensitivity and simplicity but can only be used to detect related members of known virus families. Subtractive hybridisation techniques, such as RDA, are technically more complex and time-consuming and are less sensitive but do not require any knowledge of the potential infectious agent. The recently described RCA technique can only be used to amplify circular DNA genomes but, like RDA, does not depend on any *a priori* knowledge of the sequence of the potential virus. Both degenerate PCR assays and RDA have a proven track record and RCA holds promise for the future.

The choice of method for use in virus hunting will depend on: the evidence suggesting involvement of a particular virus family or type; the index of suspicion; the reagents or samples that are available; and the predicted level of infection and therefore the required assay sensitivity. Of the assays described above, degenerate PCR assays are the most straightforward to perform and have the most applicability to screening vaccine cell substrates. These assays may prove useful in the investigation of novel cell substrates, such as clonal cell lines that are potentially tumourigenic.

Acknowledgements

We would like to thank Freda Alexander for performing the power calculations used to determine the required number of RDA representations for virus discovery and David Onions for helpful discussion. Work in our laboratory is supported by a Leukaemia Research Fund specialist programme grant.

Table 1. Primers used in degenerate PCR assays

Number	Sequence
Herpesvirus	
1A	*GACTTTCCAAGTTTC*TAYCCNAGYATHAT
1B	*GACTTTCCAAGTTTC*TAYCCNTCNATHAT
2	*ACAAACATACAGTCC*GTRTCNCCRTADAT
3A	*GTTTGATGCCGACCT*TAYGGNTTYACNGG
3B	*GTTTGATGCCGACCT*TAYGGNGTNACNGG
Polyomavirus	
4	*GTTTATGGTTGTCTTT*GANGAYGTNAARGG
5A	*GTTTCGGTGTTTCTTTT*CNARRTTNACYTT
5B	*GTTTCGGTGTTTCTTTT*CNARRTTNACNGG
6	*GTTTGAGGCACAGAA*TAYTCRTTCATNGT

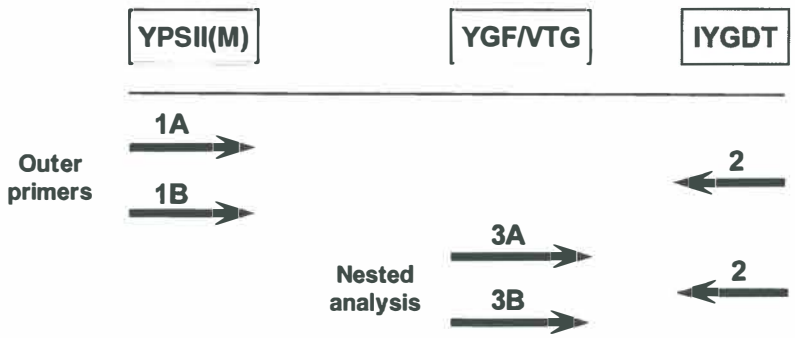
Primer numbers refer to those used in Figures 1 and 2. Clamp sequences are shown in italics.
N= A+C+G+T; Y=A+T; R=A+G; D=G+A+T; H=A+T+C.

Figure 1. Degenerate PCR assay for detection of herpesviruses. Panel a: Amino acid sequences of the herpesvirus polymerase proteins were aligned and blocks of conserved sequence identified – the alignment of the human herpesviruses is shown but these sequences were well conserved across herpesviruses of all species except fish. Pentapeptides were then selected (boxed sequences) on the basis of codon usage, to minimise degeneracy of the corresponding primer. Panel b: Three outer primers and two inner primers were synthesised; primer 1 was split into two syntheses, which differed in their codon usage for serine, and primer 3 was split in two syntheses with primer 3A including all nucleotide sequences encoding phenylalanine (F) and 3B including those encoding valine (V). The assay is run in a semi-nested format using the indicated primers. Panel c: GeneScan analysis showing amplification of Epstein-Barr virus (EBV) specific *pol* gene sequences using outer primers 1A and 2. * indicates size markers. Panel d: GeneScan analysis of products of semi-nested assay showing detection of EBV *pol* sequences using primers 3A and 2. * indicates size markers.

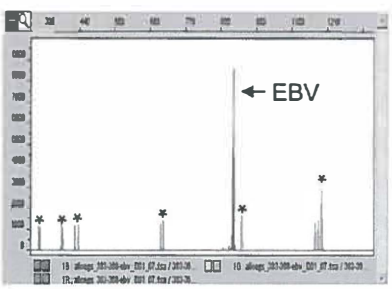
a

HSV1	ASLYPSIIQAHNL	VYGFTGV	IYGDTDS
HSV2	ASLYPSIIQAHNL	VYGFTGV	IYGDTDS
VZV	ASLYPSIIQAHNL	VYGFTGV	IYGDTDS
EBV	ASLYPSIIQAHNL	VYGFTGV	IYGDTDS
HCMV	ASLYPSIMQAHNL	FYGFTGV	IYGDTDS
HHV-6	QSLYPSIMQAHNL	VYGVTGA	IYGDTDS
HHV-7	QSLYPSIMQAHNL	VYGVTGA	IYGDTDS
HHV-8	ASLYPSIIQAHNL	VYGFTGV	IYGDTDS

b



c



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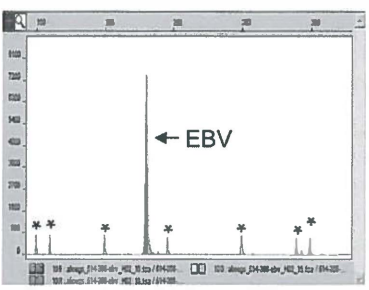
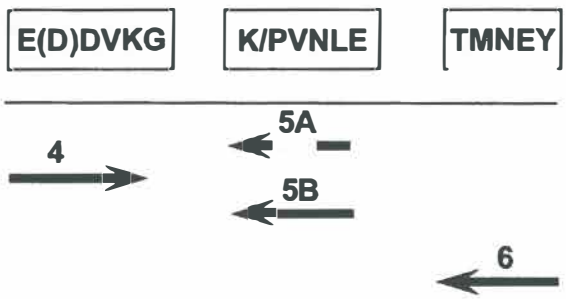


Figure 2. Degenerate PCR assay for detection of polyomaviruses: Panel a: Amino acid sequences of the polyomavirus large T antigen were aligned and blocks of conserved sequence identified (boxed areas). Panel b: One 5' primer and three 3' primers were synthesised; primer 5A covers all possible codons for KVNLE and primer 5B covers all possible codon usage for PVNLE. Primer 6 is derived from the sequence TMNEY; this sequence is not conserved across all polyomaviruses but the corresponding primer has a degeneracy of only 16-fold, resulting in a sensitive PCR reaction. Panel c (upper electropherogram): GeneScan analysis showing detection of bovine polyomavirus (BPyV) using primers 4 and 5B. Panel c (lower electropherogram): GeneScan analysis showing detection of SV40 sequences using primers 4 and 6. * indicates size markers.

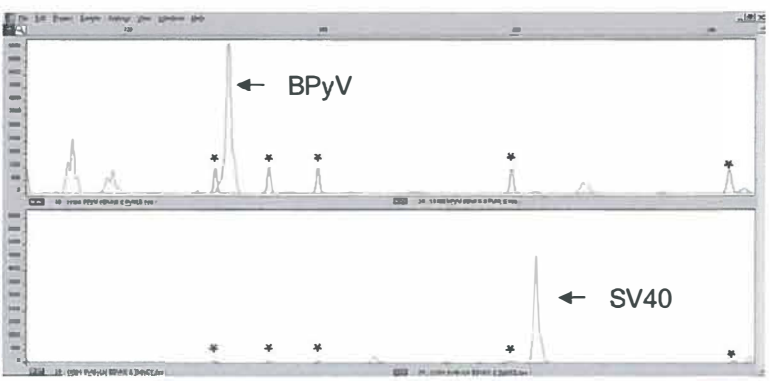
a

```
                    VFEDVKGT    SVKVNLEKKHLNKRTQIFPPGLVTMNEY
                    VFEDVKGT    SVKVNLERKHQNKRTQVFPPGIVTMNEY
                    VFEDVKGT    SVKVNLEKKHLNKRTQIFPPGIVTMNEY
                    VFEDVKGK    AVPVNLERKHQNKVTQIFPPGIVTCNEY
Budgerigar fledgling LFEDVKGT    SVPVNLERKHQNKVSQIFPPGIITMNNY
Goose hemorrhagic   LFEDVKGQ    AVPVNLERKHQNKVSQIFPPGIITMNHY
                    VFEDVKGQ    SVKVNLEKKHVNKRSQIFPPCIVTANEY
Murine strain a2    CFEDVKGQ    SVKVNLEKKHSNKRSQLFPPCVCTMNEY
Murine strain a3    CFEDVKGQ    SVKVNLEKKHSNKRSQLFPPCVCTMNEY
                    LLDDVKGQ    TIKVNLEKKHVNKRSQIFPPVIMTMNEY
                    CVEVLAEK    AVKVNLEKKHLNKKTQIFPPGIVTSNEY
```

b



c



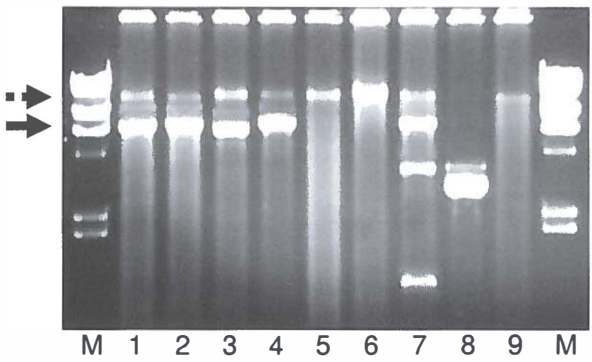


Figure 3. Analysis of equine sarcoid tumour samples using rolling circle amplification: DNA samples were subjected to rolling circle amplification followed by restriction enzyme digestion with *Hin*dIII and agarose gel electrophoresis. Solid arrow indicates the band representing the complete papillomavirus genome; dashed arrow indicates non-specific products. Lane M, *Hin*dIII-digested lambda phage DNA size marker; lanes 1-5, samples from equine sarcoid tumour samples – full length papillomavirus genomes are detected in four of the samples; lane 6, equine DNA; lane 7, cloned genome of bovine papillomavirus type 1 in a DNA background; lane 8, plasmid ; lane 9, water.

Chapter 7

Serial analysis of gene expression of primary material from Hodgkin lymphoma

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Chapter 7

Abstract

Classical Hodgkin lymphoma (cHL) is one of the commonest lymphomas in the Western world. The aetiology of cHL is not completely understood largely because the malignant cells, the Hodgkin and Reed-Sternberg (HRS) constitute only 1% of the tumour mass, and this has hindered their characterisation. Epstein-Barr virus (EBV) is causally associated with a proportion of cHL cases and epidemiological data suggest that an infectious agent may be involved in the remaining cases, although this agent remains elusive. The generation of gene expression libraries from primary HRS cells may enable identification of genes involved in the disease pathogenesis. The primary aim of this study was to identify genes that are differentially expressed in HRS cells and their 'normal' counterpart. Serial Analysis of Gene Expression (SAGE) libraries were generated from CD30-positive cells enriched from an EBV-positive and EBV-negative case of cHL, as well as CD77-positive cells from a reactive lymph node. Among the genes that had increased expression in the cHL cases compared with the "normal" counterpart were Protein Kinase C eta and Galectin 2. Results from specific relative quantitative PCR for these genes showed that both Protein Kinase C eta and Galectin 2 were indeed present at higher levels in the cDNA from the cHL samples used to generate these libraries. Although these data are preliminary, further investigation of cHL cases is warranted to determine whether these genes are involved in the pathogenesis of cHL.

Introduction

Hodgkin lymphoma (HL) is a malignant disease which accounts for around 30% of all lymphomas. It is subdivided into classical Hodgkin lymphoma (cHL) which accounts for around 95% of all cases, and noduar lymphocyte predominance HL (NLPHL) which makes up the remainder (WHO 2001). NLPHL is recognised as a distinct disease entity (Harris 1999) and the focus of this work is on cHL. cHL is characterised by the presence of Hodgkin and Reed-Sternberg (HRS) cells, the proposed malignant cells of the disease, in a polymorphous cellular background. A striking feature of cHL is the scarcity of the malignant cells, which usually constitute less than 1% of the cells within the tumour mass. The rarity of these cells has hindered their characterisation and our understanding of this disease. Advances in molecular techniques over the last decade have established that the HRS cells are of B-cell origin in the majority of cHL cases as evidenced by the detection of immunoglobulin (Ig) gene rearrangements in isolated HRS cells (Kuppers *et al.,* 1994;Kanzler *et al.,* 1996b;Marafioti *et al.,* 2000). The majority of cHL cases show evidence of somatic hypermutation of variable (V) regions of Ig genes indicating that they have participated in a germinal centre (GC) reaction; however, all HRS cells from an individual case show the same hypermutation pattern, therefore somatic hypermutation has ceased (Brauninger *et al.,* 2006). In about 25% of cases crippling mutations which render the Ig rearrangements non functional are detected (Kanzler *et al.,* 1996b;Kuppers 2002). Even in the absence of crippling mutation, there is down-regulation of Ig expression thus suggesting that the HRS cells in most cases are derived from pre-apoptotic GC B cells.

The aetiology of cHL is unknown although EBV is causally associated with a proportion of cHL cases (IARC 1997). Epidemiological data suggest that an infectious agent may be involved in EBV-negative cHL cases particularly those aged 15-34 years and of nodular sclerosis (NS) subtype. It has been suggested that EBV may be using a hit and run mechanism in these cases although the available data do not support this idea (Gallagher *et al.,* 2003). We, and others,

have looked for genomes of lymphotropic and/or oncogenic viruses other than EBV in HL biopsies with negative results (Jarrett *et al.,* 1988;Khan *et al.,* 1993;Secchiero *et al.,* 1998;Berneman *et al.,* 1998;Armstrong *et al.,* 1998b;Gallagher *et al.,* 2002;MacKenzie *et al.,* 2003;Wilson *et al.,* 2006).

A number of studies have attempted to determine the overall gene expression profile of HRS cells, the majority of which have used cHL-derived cell lines (Kapp *et al.,* 1999;van den Berg *et al.,* 1999;van den Berg *et al.,* 2000;Kuppers *et al.,* 2003;van den Berg *et al.,* 2003;Schwering *et al.,* 2003a;Schwering *et al.,* 2003b;Janz *et al.,* 2006;Staber *et al.,* 2006) or whole cHL tissue (Devilard *et al.,* 2002;Sanchez-Aguilera *et al.,* 2006). An advantage of using HL-derived cell lines for investigative studies is that the material is abundant and readily available; however, all of the cell lines have been derived from patients with end-stage disease and may not, therefore, be truly representative of primary HRS cells. In addition, only the L1236 cell line has actually been shown to be clonally related to original tumour material (Kanzler *et al.,* 1996a).

The use of Serial Analysis of Gene Expression (SAGE) demonstrated the extremely high expression of the chemokine TARC (CCL17) in HL-derived cell lines and suggested a possible role in attracting Th2 cells to the surrounding infiltrate (van den Berg *et al.,* 1999). In a subsequent SAGE study, expression of the B-cell receptor inducible gene BIC was identified in all subtypes of HL (van den Berg *et al.,* 2003) and this led to the investigation and detection of the mature microRNA-155 in HL-derived cell lines and tissue samples (Kluiver *et al.,* 2005). In comprehensive studies using SAGE and cDNA microarrays, the loss of the B-lineage specific gene expression program was demonstrated in HL-derived cell lines (Kuppers *et al.,* 2003;Schwering *et al.,* 2003a;Schwering *et al.,* 2003b).

The first study to use primary HRS cells for global gene expression profiling generated cDNA libraries from micromanipulated HRS cells from a case of nodular sclerosis (NS) HL and a case of NLPHL (Cossman *et al.,* 1999). In addition,

libraries were generated from two HL-derived cell lines, L428 and KMH2. For final analysis of the libraries, the authors combined the sequences obtained from both HL cases and the HL-derived cell lines. The expression profile supported a B-cell lineage for HRS cells, but subsequent studies have shed doubt on whether the 'primary tumour' cells were bona fide HRS cells. Further global gene expression studies on primary HRS cells are therefore necessary.

The aim of the present study was to generate SAGE libraries from HRS cells away from the reactive infiltrate of the tumour, in order to identify known or novel genes that are involved in the pathogenesis of cHL. The primary aim was to determine differences in gene expression between HRS cells and a "normal" counterpart for further investigative analyses. Secondary aims were to compare expression differences between EBV-positive and negative HRS cells and also to identify novel viral transcripts.

Material and Methods

Sample selection and processing: Excess material from diagnostic lymph node biopsies was mechanically disrupted and mononuclear cells enriched by gradient centrifugation using Lymphoprep™ (Life Technologies, Paisley, UK). Analytical flow cytometry was performed using antibodies to a panel of markers including CD30 and the remaining cell suspension was stored viably in liquid nitrogen. Two cases of cHL were selected for inclusion in this study on the basis of availability of sufficient cells and the presence of an identifiable population of CD30-positive cells on flow cytometry. Case 6656 was a 15 year old female with a non-EBV-associated tumour and a diagnosis of NSHL. Case 5874 was a 70 year old male with an EBV-associated tumour, also of the NS subtype. Case 6333 was a 26 year old male with benign lymphadenopathy. The HL-derived cell line L428 was included for subsequent comparison.

Cell sorting: Cell sorting was performed using an EPICS Elite flow cytometer (Beckman Coulter, High Wycombe, UK). Single cell suspensions from cHL samples were stained with the BerH2 anti-CD30 murine monoclonal antibody (Dako UK Ltd, Cambridgeshire, UK) followed by a phycoerythrin-conjugated polyclonal goat anti-mouse Ig/F(ab')2 (Dako UK Ltd). HRS cell fractions were selected on the basis of CD30-positivity and forward and side scatter characteristics (Figure 1). Following cell sorting, a small aliquot of cells from the positive fraction was analysed by flow cytometry to assess purity. In the positive fraction from case 6656, 86.2% of events in the total ungated population were CD30-positive and 3 x 10^5 cells were available for downstream analysis. For 5874, 86.8% of the events in the ungated positive fraction were CD30-positive and 1.2 x 10^5 cells were recovered.

GC B-cells were chosen as the 'normal' counterpart of HRS cells for comparison, as HRS cells are thought to be derived from pre-apoptotic GC B-cells (Kuppers 2002). In order to enrich GC B-cells, a single cell suspension from a reactive lymph node was stained sequentially with an anti-CD77 monoclonal antibody (Immunotech, Buckinghamshire, UK) and a phycoerythrin-conjugated goat anti-rat secondary antibody (Immunotech). Following sorting of CD77-positive events falling within the 'lymphoid' gate, the positive fraction contained 3.1 x 10^5 cells with a purity of 93%.

RNA extraction: RNA extraction was performed immediately following cell sorting to preserve the quality of RNA. Total RNA was isolated using the RNAqueous™ kit (Ambion, Cambridgeshire, UK) according to the manufacturers protocol, and RNA was eluted in a total volume of 25 µl.

cDNA synthesis: cDNA synthesis was performed using 3 µl of eluted RNA, roughly equivalent to 180 ng. Since insufficient RNA was available for the conventional SAGE protocol (Velculescu *et al.,* 1995), the SMART cDNA kit (BD

Clontech, Oxford, UK) was used for cDNA amplification prior to SAGE. This methodology affords amplification of cDNA whilst maximising the length of mRNA transcripts. In conventional SMART the first strand cDNA is synthesised by using an oligo dT SMART CDS primer and a SMART IIA oligonucleotide that facilitates a switch mechanism at the 5' end of the mRNA sequence and enables longer transcripts to be synthesised. The amplification PCR which follows uses a single primer which is homologous to common sequences in both the oligo dT SMART CDS primer and SMART IIA oligonucleotide. SAGE requires the identification of a 10 bp specific gene tag which lies immediately 3' to the most 3' Nla III site in the cDNA derived from poly (A) mRNA; thus, primers used in any prior amplification step must distinguish the 5' and 3' ends of the cDNA. The SMART primers were therefore modified, as shown below, and for long-distance (LD) PCR the 3' LD primer was biotinylated to allow capture on immobilised streptavidin. The number of PCR cycles was optimised for each sample to ensure maximum yield of cDNA whilst not over-amplifying the samples.

***Ist Strand CDNA synthesis*:**

Modified SMART II A oligonucleotide
5-'AAGCAGTGGTATCAACGCAGAGATCGCGGG-3'
Modified 3' SMART CDS Primer II A
5'-GAGTCCAGAAGAGTGCGATGAGTACT$_{(30)}$VN-3'

***cDNA Amplification by LD PCR*:**

Modified 5' PCR Primer II A
5'-AAGCAGTGGTATCAACGCAGAGA-3'
Biotinylated 3' primer for capture of polyA RNA (5' label)
5'-GAGTCCAGAAGAGTGCGATGAG-3'

SAGE: Amplified cDNA samples were double purified by resin binding and column purification using the QuickStep™ 2 PCR Purification kit (Edge BioSystems, Gaithersburg, USA). The biotinylated cDNA was bound to DYNAL M-280 streptavidin beads (Invitrogen, Paisley, UK) and SAGE libraries were generated using the I SAGE kit (Invitrogen), essentially as described by the manufacturer.

Nucleotide sequencing: Cloned libraries were transformed into DH5α cells (Invitrogen) and individual bacterial colonies diluted in 20 µl of H_20. Two µl of this diluent was used as template in a Rolling Circle Amplification reaction (Templiphi™ kit, Amersham plc, Buckinghamshire, UK) prior to sequencing. Nucleotide sequencing was performed using the Big Dye Terminator™ cycle sequencing reaction kit V3.1 (Applied Biosystems, Warrington, UK). Excess dye terminators were removed using Performa™ v3-96 Well Short Plates (Edge BioSystems) and products resuspended in 25 µl of Hi-di Formamide (Applied Biosystems). Sequencing reactions were analysed on an Applied Biosystems 3100 Genetic Analyzer using DNA sequencing Analysis software™ (Applied Biosystems).

SAGE Tag Analysis: Sequencing files were analysed using the SAGE 2000 software v4.5 kindly provided by Dr KW Kinzler (Johns Hopkins Oncology Center, Baltimore, Maryland, USA). Tags were normalised to 29898 (tag count of 6333) with duplicate di-tags and linker tags removed. Results were linked to the SAGEmap human database (http://www.ncbi.nlm.nih.gov/projects/SAGE/) and the CGAP best gene for a tag map (http://cgap.nci.nih.gov/SAGE) to identify the corresponding genes. The CGAP best gene was chosen for subsequent analysis because this designates one gene hit per SAGE tag. Tags with no gene match were also checked on the Tagmapper database (http://tagmapper.ibioinformatics.org/). Further comparisons were performed using Microsoft Access and Excel programmes. Genes that were up and down regulated in the cHL cases compared to the GC sample were analysed. We also looked for expression differences in the EBV-negative cHL case compared to the EBV-positive case. Genes were considered differentially expressed if either the tag

count showed a ≥ 5-fold increase or decrease, or there was a tag count of ≥ 5 in one normalised library but no tags in another.

Relative Quantitative PCR: The biotinylated SMART cDNA used to generate the SAGE libraries was also used as template for quantitative reverse transcriptase (qRT) PCR in order to validate the SAGE results. SMART cDNA previously generated from cHL case 6656 using the conventional SMART protocol was also assessed for comparison. In addition, SMART cDNA was available from a similarly enriched HRS cell population from another EBV-negative case (6214). A β-actin MGB PDAR™ assay was used as an endogenous control and cDNA samples were diluted to bring them into the dynamic range for this assay. Two genes chosen for investigation were Protein kinase C (PKC) eta and Galectin 2. All assays were commercially available (Applied Biosystems, Warrington, UK). Amplification and relative quantitation were performed using an Applied Biosystems 7500 Real Time PCR system (Applied Biosystems). Results were calibrated to the GC (6333) result and relative quantitation calculated by comparison to the β-actin endogenous control and a standard ΔΔCt method. Relative expression differences were then calculated using the formula $2^{-\Delta\Delta Ct}$ calculation.

Results

SAGE analysis: Table 1 shows the number of SAGE tags generated from each of the samples and the corresponding duplicate di-tag count.

Table 1: Total Tag counts

Case	Total tags	Duplicate di-tags
6656 (EBV-ve HL)	5282	6025
5874 (EBV+ve HL	45568	2164
6333 (GC B-cells)	29898	1518

The libraries from both 6333 (GC) and 5874 (EBV-positive HL case) are satisfactory with a low number of duplicate di-tags. This is an indication that further sequencing will provide additional information; however, the 6656 library (EBV-negative HL case) yielded relatively few tags before the duplicate di-tag number indicated that further sequencing would not yield additional informative data. Although the 6656 library is included in some of the comparisons, this rather small library will only contribute information on highly expressed transcripts. The 6656 library was not included in the analyses of down-regulated genes. The unique tags identified are shown in Table 2.

Table 2: Unique tags identified

Case	Unique tags	Gene hits	No match
6656 (EBV-ve HL)	725	581	144
5874 (EBV+ve HL)	3580	2894	686
6333 (GC B-cells)	2404	1893	511

Differential expression: Analysis of the normalised result revealed that forty nine tags were increased ≥ 5-fold in 5874 compared to 6333. Twenty five of these were also increased in 6656. Thirty three tags were present with a count of ≥ 5 in 5874 but not present in 6333. Twenty of these were also present in 6656. One hundred and seven tags were decreased ≥ 5-fold in 5874 compared to 6333. Seventy eight tags had a count of ≥ 5 tags in 6333 but were absent in 5874.

One of the genes highly expressed in both cHL cases when compared to the GC library was the gene for the chemokine CCL17, which has previously been shown to be highly expressed in HL-derived cell lines by the SAGE technique (van den Berg *et al.,* 1999). Other genes with increased expression in one or both libraries were: CD44; Fascin; Neuron-specific enolase 2; Cathepsin B and Survivin. A list of

the 10 genes most highly expressed in case 5874 compared to 6333 are shown in Table 3. The 10 most highly expressed genes in 6656 compared with 6333 are shown in Table 4 and the genes most highly expressed in 5874 with no gene tags present in 6333 are shown in Table 5 along with the corresponding tags for 6656. Primary analysis was focussed on the validation of known genes that were differentially expressed in these libraries. Two genes chosen for further analysis from the list shown in Table 4 were PKC eta and Galectin 2 because they were present at greater than 5 tags in both cHL cases following normalisation and not present at all in 6333 (GC). From what is known about these genes, they may have a plausible function in HL.

No EBV tags were detected in the library from the EBV-positive case 5874, despite the demonstration that EBV latent gene products were easily detectable in the starting cDNA sample (data not shown). A number of tags had no gene hits and may therefore be novel gene/viral sequences or splice variants.

Relative Quantitative PCR: Following qRT-PCR on the biotinylated cDNA, which was also used to generate the SAGE libraries, the relative quantitation between the cHL samples (5874 and 6656) and GC sample (6333) as calibrator were assessed using the ΔΔCt method. Relative expression differences were then calculated using the formula $2^{-\Delta\Delta Ct}$ calculation. The results shown in Figure 2 indicate that both cHL cases have increased expression of PKC eta and Galectin 2 compared to 6333 (GC).

Discussion

This study describes the generation of the first SAGE libraries from primary HRS cells. To date we have focused on analysing and validating gene expression differences between the cHL and GC libraries. One of the genes that was most highly differentially expressed in both cHL samples when compared to the GC

library was CCL17, which had been shown previously to be highly expressed in HL cell lines and HL tissue samples (van den Berg *et al.,* 1999). Similar to other gene expression studies of cHL, fewer genes were up-regulated than down-regulated in HRS cells compared to GC B-cells (Schwering *et al.,* 2003a;Kluiver *et al.,* 2006). A number of gene transcripts were up-regulated that had previously been shown to be expressed in HL by SAGE (Schwering *et al.,* 2003a). These included: CD44; Fascin; Neuron-specific enolase 2 and Cathepsin B. Survivin, which has also been shown to be highly expressed in HL using tissue microarrays, was also increased in our EBV-negative cHL library (Garcia *et al.,* 2003). Initial comparisons with previous studies therefore suggest that these libraries are representative of bona fide HRS cells.

Although the above results indicate that these libraries will be a useful resource for further analyses, they have certain limitations. HRS cells are notoriously difficult to enrich as they are extremely fragile and the purity of our enriched fractions was only ~86%. In addition, the small amounts of RNA generated from these purified cell populations necessitated the inclusion of the SMART amplification procedure prior to generation of the SAGE libraries. It is possible that this could result in a bias towards small transcripts; however, libraries showed no obvious over-representation of tags from small transcripts (data not shown). A previous study, also using a SMART based methodology for amplification of cDNA prior to SAGE, showed that the amplification by PCR had not distorted the representation of their library (Neilson *et al.,* 2000). A comparative evaluation of amplification techniques for expression profiling at the single-cell level concluded that SMART PCR amplification correlated best with non-amplified cDNA at a global level compared to T7-based *in vitro* transcription and global PCR amplification (Subkhankulova and Livesey 2006). The literature therefore supports the use of the SMART amplification method for this type of analysis. To determine whether the use of the SMART amplification has biased the representation of our libraries, we constructed a SMART-amplified SAGE library from the HL-derived cell line L428. We are currently constructing a library using non-amplified cDNA from L428 for

comparison. This will enable us to validate the amplification procedure and further extend our investigations of the chosen genes of interest if the libraries are comparable.

Initial analysis identified differential expression of Galectin 2 and PKC eta, both of which had increased expression in the cHL SAGE libraries compared to the GC library. qRT PCR assays performed using the original cDNA from these cases as template confirmed these findings. At present we are optimising immunohistochemical assays for Galectin 2 and PKC eta to determine if the encoded proteins are expressed by HRS cells in other diagnostic samples. In addition, we will perform qRT PCR on cDNA samples from HRS cells obtained by either laser microdissection or enrichment, and also on various B-cell populations.

Galectins, which are a family of carbohydrate-binding proteins, are novel regulators of immune cell homeostasis (Rabinovich *et al.,* 2002;Sturm *et al.,* 2004;Liu and Rabinovich 2005;Toscano *et al.,* 2006). Galectins influence the development of the T-cell immune response including cytokine secretion, T-cell survival and activation (Bianco *et al.,* 2006). Galectin 1 has been shown to counteract the pathogenic effect of Th1 cells in autoimmune retinal disease and skew the immune response towards a Th2 and T-regulatory profile in vivo (Toscano *et al.,* 2006). This is of interest in cHL as the reactive infiltrate surrounding the HRS cells contains T cells that are primarily Th2 and T-regulatory cells. It is postulated that the Th2 cells may be attracted to the surrounding infiltrate in cHL by secretion of the chemokine TARC by HRS cells (van den Berg *et al.,* 1999). Increased expression of Galectin 1 has been demonstrated previously in HL-derived cell lines (Schwering *et al.,* 2003a;Kluiver *et al.,* 2006) but increased expression of Galectin 1 was not detected in our SAGE libraries from enriched HRS cells. Although structurally similar to Galectin 1, Galectin 2 expression is mainly confined to the gastrointestinal tract. Galectin 2 has also been shown to shift the cytokine secretion pattern of activated T cells to a Th2 profile and is also an inducer of T cell apoptosis (Sturm *et al.,* 2004). It is possible that Galectin 2 expression by the HRS

cells is contributing to the phenotype of the T cells within the reactive infiltrate of the HL tumour and is creating a favourable environment for HRS cell survival.

PKC is a family of 12 serine/threonine kinases and several of its isoforms have been implicated in the regulation of apoptosis. Inactivation of PKCs sensitises tumour cells to drug-induced apoptosis while overexpression confers protection (Cartee and Kucera 2000). PKC eta, a novel PKC, has been shown to be involved in drug resistance and regulation of apoptosis in a number of human cancers including breast cancer (Beck *et al.,* 1998a);(Masso-Welch *et al.,* 2001), ovarian cancer (Beck *et al.,* 1998b) and colon cancer (Doi *et al.,* 1994). Many studies have also shown that PKC eta is an effective inhibitor of apoptosis. In human keratinocytes PKC eta inhibits UV-induced activation of caspase 3 (Matsumura *et al.,* 2003) and in prostate cancer cells down-regulation of PKC eta potentiates the cytotoxic effects of tumour necrosis factor-related apoptosis-inducing ligand (Sonnemann *et al.,* 2004b). In addition, the down-regulation of PKC eta by antisense oligonucleotides sensitised lung cancer cells to the effects of anti-neoplastic drugs (Sonnemann *et al.,* 2004a).

The exact mechanism responsible for the survival of HRS cells in cHL is still unclear, although a number of factors are known to be involved in their rescue from apoptosis, including expression of nuclear factor-κB (NF-κB). NF-κB is a transcription factor that regulates the expression of many effector molecules including growth factors, cytokines and anti-apoptotic proteins in response to ligation of surface receptors (Bonizzi and Karin 2004). It is now widely accepted that constitutive activation of NF-κB in HRS cells plays a critical role in escape from apoptosis (Bargou *et al.,* 1997). Both cFLIP and XIAP are NF-κB target genes and therefore constitutive NF-κB activity in HRS cells may well be responsible for the anti-apoptotic phenotype of the HRS cells (Re *et al.,* 2005). There are several mechanisms of activation of NF-κB in cHL which include EBV LMP-1 expression, c-rel amplification, IκBα mutation/deletion and constitutive CD30 signalling (Emmerich *et al.,* 1999;Cabannes *et al.,* 1999;Horie *et al.,* 2002;Joos *et al.,*

2003;Emmerich *et al.,* 2003;Grimm *et al.,* 2005;Osborne *et al.,* 2005;Uchihara *et al.,* 2006). In addition to CD30, CD40 (Gruss *et al.,* 1994) and RANK (Fiumara *et al.,* 2001) are two other members of the TNF receptor family that are expressed on HRS cells and may contribute to HRS cell survival by activating NF-κB. Overexpression of PKC eta may also contribute to HRS cell survival and could represent a novel therapeutic target.

A secondary aim of the present study was to compare the libraries generated from the EBV-positive and EBV-negative cHL cases in order to identify viral transcripts present in the EBV-negative HRS cells. EBV tags were not detected in the SAGE library from case 5874 (EBV-positive case). EBV latent gene transcripts were detected in cDNA from this case using quantitative PCR; this indicates that very large numbers of tags must be sequenced in order to detect EBV viral transcripts. The library generated from 5874 is far from exhausted and we intend to increase the tag numbers on this case by continuing sequencing analysis. Larger SAGE libraries are certainly preferable for the identification of genes present at low copy number. Indeed, it has been shown that increasing the number of sequenced tags, not only enhances the reproducibility of the SAGE technique for the detection of known genes, but also increases its power for the possible detection of novel transcripts (Dinel *et al.,* 2005). The library generated from case 6656 (EBV-negative case) was small although this library will add data on highly expressed genes. A number of transcripts were detected in the SAGE library from this case that had previously been shown to be highly expressed in HRS cells. In both cHL libraries a number of tags had no gene hits and could represent novel splice variants, novel genes or viral sequences.

Further analyses on these SAGE libraries from sorted HRS cells are required to maximise the potential of this valuable material for the discovery of known or novel genes that may be involved in the pathogenesis of cHL.

Table 3. Tags increased ≥ 5-fold in SAGE library from case 5874 (cHL) compared to 6333 (GC).

Tag Sequence	5874/6333	Symbol	Description
GGCACAAAGG	70	CCL17	Chemokine (C-C motif) ligand 17
GTGCACTGAG	28.5	HLA-A	Major histocompatibility complex, class I, A
GCAAAAAAAA	24	PDXK	Pyridoxal (pyridoxine, vitamin B6) kinase
TTTGAAATGA	23	SAT	Spermidine/spermine N1-acetyltransferase
TCTACACGTG	19	PFC	Properdin P factor, complement
TGGGTGAGCC	14	CTSB	Cathepsin B
CTCTTCGAGA	10	GPX1	Glutathione peroxidase 1
GTGCTGGACC	9	PSME2	Proteasome (prosome, macropain) activator subunit 2 (PA28 beta)
GGGGCTGGAG	9	PLXND1	Plexin D1
GAAGAACAAG	8.5	SAT	Spermidine/spermine N1-acetyltransferase

Column 5874/6333 shows the fold increase in 5874 compared to 6333. SAGE tags and corresponding gene hits are shown for the top 10 differentially represented genes.

Table 4. Tags increased ≥ 5-fold in SAGE library from case 6656 (cHL) compared to 6333 (GC).

Tag Sequence	6656/6333	Symbol	Description
GCCCAGCTGG	82	EEF1D	Eukaryotic translation elongation factor 1 delta
GGCACAAAGG	67	CCL17	Chemokine (C-C motif) ligand 17
TTTGAAATGA	50	SAT	Spermidine/spermine N1-acetyltransferase
GAAGAACAAG	45	SAT	Spermidine/spermine N1-acetyltransferase
TGGGTGAGCC	39	CTSB	Cathepsin B
ATCACGAAGG	39	VAV2	Vav 2 oncogene
TATCTGTCTA	33	SET	SET translocation (myeloid leukemia-associated)
TGCCCTTCGG	28	ZNF342	Zinc finger protein 342
TCTCTATTAA	28		Transcribed locus, moderately similar to XP_371398.2 myosin
TCTGCAAAGG	23	HMGB2	High-mobility group box 2

Column 6656/6333 shows the fold increase in 6656 compared to 6333. SAGE tags and corresponding gene hits are shown for the top 10 differentially represented genes.

Table 5. Tags present ≥ 5-fold in SAGE library from case 5874 (cHL) and not present in 6333 (GC) library.

Tag Sequence	5874	6333	6656	Symbol	Description
TCAGTGACCA	32	0	5	PRKCH	Protein kinase C, eta
TCCTCTTTCA	26	0	62	LGALS2	Galectin 2
CCCACAACCT	26	0	16	FCN1	Ficolin 1
CCCGTCCGGA	18	0	39	RPL13	Ribosomal protein L13
GAGAACCGTA	13	0	0	NPDC1	Neural proliferation, differentiation and control, 1
GTGTGCGCCT	11	0	0	CARD8	Caspase 8
AAATCTCTGG	9	0	16	PREX1	Phosphatidylinositol 3,4,5-trisphosphate- exchanger 1
TTCAATAAAA	8	0	11	RPLP1	Ribosomal protein, large, P1
GGACCTGCGC	7	0	16	RNASET2	Ribonuclease T2
GGAGGTTGAA	7	0	5	EDG8	Endothelial differentiation, sphingolipid G-protein-coupled receptor, 8

SAGE tags and corresponding gene hits are shown for the top 10 differentially represented genes in 5874 and not expressed in 6333. The corresponding tags for the 6656 library are also shown.

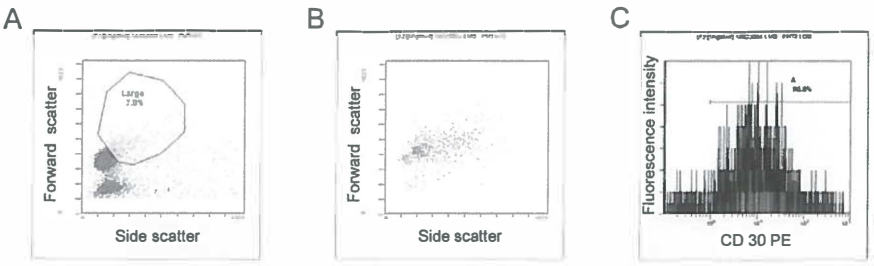

Figure 1. Enrichment of Hodgkin and Reed-Sternberg cells from case 5874. (A) Scatter plot of original sample showing gate used in subsequent sort. (B) Scatter plot following cell sorting. (C) Purity of enriched sample as assessed by CD30-positivity.

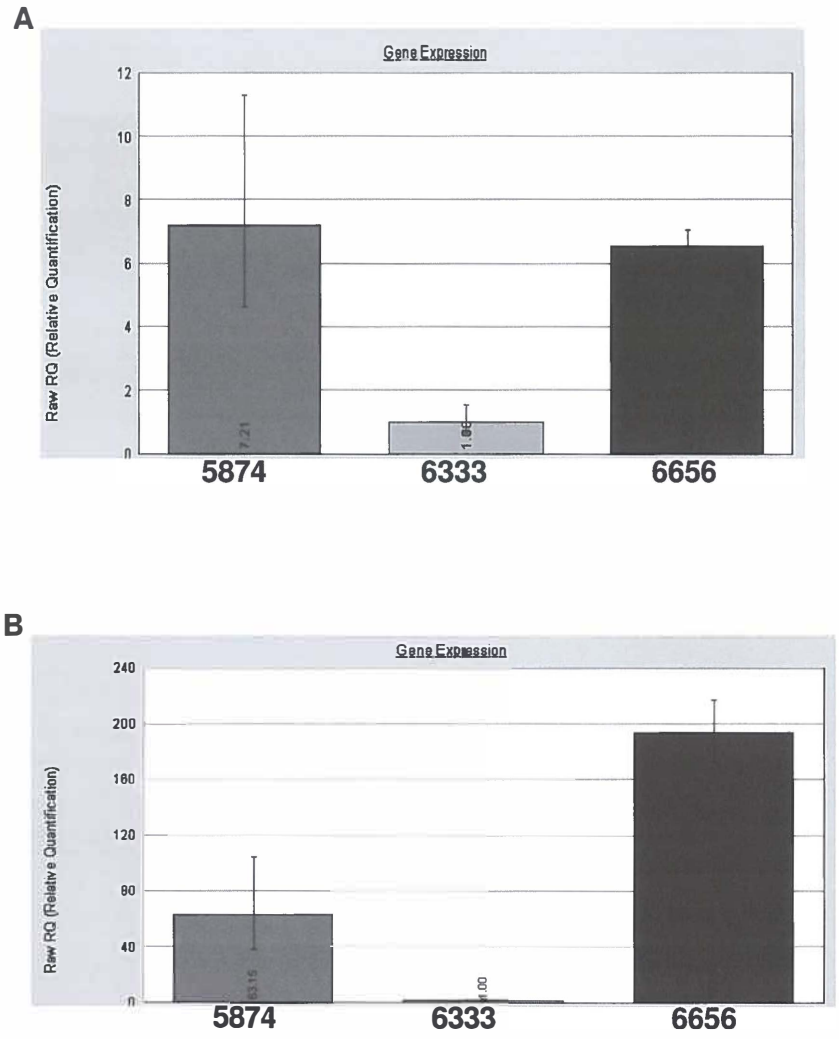

Figure 2. Relative quantitative expression differences between cHL cDNA (5874 and 6656) compared with the GC cDNA (6333). (A) PKC eta gene expression assay. (B) Galectin 2 gene expression assay.

Chapter 8

Discussion and Future Plans

Chapter 8

Discussion and Future Plans

The mechanism of transformation of the HRS cells of cHL is not completely understood. EBV is detectable in HRS cells, the tumour cells of cHL, in around one third of HL cases in western countries and is thought to play a role in the disease pathogenesis in these cases (Long *et al.,* 1974;Armstrong *et al.,* 1992;Jarrett *et al.,* 1996;IARC 1997). The ability to detect EBV DNA in serum samples from patients with EBV-associated HL could prove useful in both clinical and epidemiological studies. Overall, the results obtained in chapter 2 suggest that EBV DNA is detectable in serum of most, if not all, patients with EBV-associated HL. In contrast, EBV DNA is infrequently found in serum from non-EBV-associated cases and, in this study, was never detected in samples from healthy individuals. Results obtained from the post-treatment samples analysed suggests that detection of EBV may be a useful prognostic marker. In a small comparative study the results also suggested that serum is superior to plasma as a source of DNA.

These results add to a growing body of data indicating that serum samples from cancer patients provide a useful source of DNA suitable for molecular analyses (Leon *et al.,* 1977;Steinman 1979;Vasioukhin *et al.,* 1994;Nawroz *et al.,* 1996;Anker *et al.,* 1997). We demonstrated that EBV DNA is present as naked DNA and not in virions in the serum of HL patients. A study of the molecular characterisation of circulating EBV DNA in the plasma of nasopharyngeal carcinoma and lymphoma patients confirmed our findings (Chan *et al.,* 2003). We suggested that this assay could prove useful in epidemiological studies and in the clinical follow-up of patients with EBV-associated HL and other EBV-associated malignancies. The monitoring of EBV DNA levels in blood has now been shown to be useful in the management of post-transplant lymphoproliferative disease (Wagner *et al.,* 2002). In addition, rising serum EBV DNA levels preceded the clinical detection of NHL by months in a HIV-infected patient (Fleisch *et al.,* 2005). Wagner and colleagues reported EBV DNA detection in the plasma of paediatric HL patients and suggested this might be of value for non-invasive diagnostic,

prognostic and follow-up tests for HL (Wagner *et al.,* 2001). A recent study questioned the sensitivity of the EBV assay employed by Wagner and colleagues, although stated that results obtained in our study were in broad agreement with their own (Gandhi *et al.,* 2006). Preliminary results from small numbers in our study showed that serum was perhaps a better source of DNA than plasma. Gandhi and colleagues used plasma as a DNA source in their study and correctly commented that no large scale study had compared the relative sensitivities between EBV detection in serum and plasma. They conclude that EBV-DNA from plasma is a sensitive and reliable biomarker for disease evaluation in EBV-positive HL patients (Gandhi *et al.,* 2006).

Since EBV is associated with only a proportion of cHL cases it has been suggested that perhaps EBV has been involved in a hit-and–run scenario with respect to EBV-negative cases (Ambinder 2000). In chapter 3 we addressed this issue using a comprehensive combined serological and molecular approach. Analysis of EBV seroprevalence rates in an epidemiological study of young adult HL revealed that cases with EBV HRS-ve HL were more likely to be EBV seronegative than controls. Furthermore, additional studies clearly showed that some HL patients have never been infected by EBV. Quantitative PCR was used to look for the presence of deleted EBV genomes in a series of adult cases with EBV HRS+ve and EBV HRS–ve HL. Subgenomic fragments were detected in equimolar proportions. This study, therefore, found no evidence to support the idea that a hit-and-run mechanism involving EBV plays a role in the pathogenesis of HL.

Epidemiological studies suggest that an infectious agent is involved in the aetiology of young adult cHL although cases in this age group are less likely to have EBV-associated disease than cases diagnosed in early childhood or older adulthood. Since previous molecular studies had failed to find a consistent association between cHL and other candidate viruses (Jarrett *et al.,* 1988;Torelli *et al.,* 1991;Secchiero *et al.,* 1998;Berneman *et al.,* 1998;Cozen *et al.,* 1998;Armstrong *et al.,* 1998b;MacKenzie *et al.,* 2003), we investigated whether novel herpesviruses

were detectable in EBV-negative HL using a highly sensitive degenerate PCR assay as discussed in chapter 4. Despite exhaustive sequencing no novel herpesviruses were detected. Chapter 5 describes the use of both conventional and degenerate PCR strategies for the investigation of possible polyomavirus involvement in HL. No direct association between polyomaviruses and EBV-negative HL was found. It therefore appears highly unlikely that members of these two virus families are directly involved in HL disease pathogenesis. More recently measles virus has been implicated as a candidate in a proportion of HL cases (Benharroch *et al.,* 2003). These results, however, remain controversial and have not been confirmed (our unpublished data and personal communication R. Kuppers). Zur Hausen and de Villiers have put forward a hypothesis suggesting a link between TT viruses and HL. The hypothesis leaves many questions unanswered and further investigative studies on the role of TTVs in HL are required to confirm or refute this suggestion (zur Hausen and de Villiers 2005).

Chapter 6 describes currently used molecular methods for virus discovery, including degenerate PCR assays, RDA and RCA, and summarises the advantages and disadvantages of each technique. We have used RCA to successfully amplify complete papillomavirus genomes from equine sarcoid tumours (Yuan *et al.,* 2006) and complete polyomavirus genomes, including the genome of the rabbit polyomavirus. The technique is relatively simple to perform, requires no *a priori* knowledge of the likely agent, and is suitable for screening purposes. An assay such as this may be useful for the investigation of HL samples for the presence of circular viral genomes such as TT viruses.

The putative virus remains elusive but complementary techniques for virus discovery need to be explored before a direct role for viruses other than EBV in this disease can be excluded. Using available sequence data from more than 140 sequenced viral genomes, Wang and colleagues designed a DNA microarray that could potentially simultaneously detect hundreds of viruses (Wang *et al.,* 2002). The array was further expanded with the objective of creating a microarray that

could detect a wide range of known viruses and also unknown viruses (Wang *et al.,* 2003). Using this strategy a novel coronavirus associated with severe acute respiratory syndrome was identified (Ksiazek *et al.,* 2003). This "Virochip" has also been used to identify a novel gammaretrovirus in prostate tumours (Urisman *et al.,* 2006) and to detect Human parainfluenzavirus 4 infection associated with respiratory failure in an immunocompetent adult (Chiu *et al.,* 2006). The possible use of a "Virochip" for the investigation of HL samples would be an exciting prospect. Such a study may provide a definitive answer to the question of whether another virus is involved in the EBV-negative cases of HL.

In search of gene expression differences between EBV-positive and EBV-negative HL we generated SAGE libraries from enriched HRS cell populations from HL patients as described in Chapter 7. As we have a long standing interest in virus hunting in HL, one of our aims in this study was the detection of other infectious agents in non-EBV associated HL. It appears at present that the tag counts in our libraries are not sufficiently large to detect viral sequences but sequencing of tags is ongoing. The library generated from the EBV-negative case was small and exhausted at a low tag count number. A number of the genes shown to have increased expression in this case when compared to the GC B-cell library had, however, been shown previously to be upregulated in HL. This is consistent with the observation that highly abundant transcripts come through early on in the SAGE analysis. A larger library is required from an EBV-negative case for the investigation of lower abundance transcripts.

At present the libraries are suitable for the investigation of gene expression differences between HRS cells and GC B-cells. In comprehensive studies using SAGE and DNA microarrays the loss of the B-lineage specific gene expression program has been demonstrated in the HRS cells of cHL (Kuppers *et al.,* 2003;Schwering *et al.,* 2003a;Schwering *et al.,* 2003b). Down regulation of these genes affects multiple pathways in B-cells including BCR signalling. Gene expression studies using primary HRS cell material will help in the characterisation

of HRS cells and perhaps identify critical genes involved in the disease pathogenesis. Preliminary results from the study presented in Chapter 7 have highlighted a number of genes that appear to show increased expression in cHL. Among the genes that had increased expression in the cHL cases compared with the GC B-cells were Protein Kinase C eta and Galectin 2. Results from specific relative quantitative PCR for these genes showed that both Protein Kinase C eta and Galectin 2 were indeed present at higher levels in the cDNA from the cHL samples used to generate these libraries. We are at present performing confirmatory analyses of these findings, and continuing the analysis of other genes of interest that show increased expression in HRS cells.

As discussed in Chapter 7 the conventional SAGE technique has been used previously as a useful tool for the characterisation of gene expression profiles in HL cell lines and HL-derived tissue. Our study aimed to look at the gene expression profile of the HRS cells themselves and identify known and perhaps novel transcripts that may be involved in the disease process. The LongSAGE technique (Saha *et al.,* 2002) releases a larger tag from each transcript which is 21 bp in length. Studies have shown that longer tags were much more efficient for the identification of novel genes in comparison with conventional SAGE tags (Saha *et al.,* 2002;Chen *et al.,* 2002). A robust LongSAGE protocol has since been described which states that as little as 50 ng of mRNA was sufficient for library construction (Gowda *et al.,* 2004). Techniques using small amounts of starting material are attractive when dealing with HRS cells, which are scarce within the HL tumour mass. Certainly LongSAGE would be advantageous for the possibility of identifying viral transcripts in HRS cells as the longer tags can be compared to genomic databases (Saha *et al.,* 2002).

The transforming event in cHL is still unknown. Additional gene expression studies are required using primary HRS cell material to clarify the complex gene expression profile of HRS cells in cHL and identify candidates involved in the disease pathogenesis. Involvement of a transforming virus in EBV-negative cases

has not been excluded and future studies, using state-of-the-art molecular techniques for virus discovery, are warranted.

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Summary

Classical Hodgkin lymphoma (cHL) is a malignant disease characterised by the presence of Hodgkin and Reed-Sternberg (HRS) cells, the proposed malignant cells of the disease, in a polymorphous cellular background. The scarcity of the HRS cells within the tumour mass has hindered the complete characterisation of these cells although they are now considered to be derived from pre-apoptotic germinal centre B cells. The aetiology of cHL is still unknown although Epstein-Barr virus (EBV) is associated with a proportion of cases and it is now accepted that EBV is involved in the pathogenesis of these cases. EBV association rates vary with age, histological subtype and geographical locale.

Results from a number of studies have indicated that the DNA present in the serum/plasma of cancer patients is largely derived from tumour cells. We speculated that the ability to detect EBV DNA in serum samples from patients with EBV-associated HL could prove useful in both clinical and epidemiological studies. EBV DNA was detected in serum from 30/33 patients with EBV-associated Hodgkin's disease but only in 6/26 patients with non-EBV-associated disease ($p<0.001$). Samples from healthy individuals were negative and only 5/12 infectious mononucleosis samples were positive. Copy number of EBV was variable. Analysis of a small group of cases suggested that EBV-positivity in post-treatment samples correlates with risk factors indicative of a poor prognosis. Overall, the results are consistent with the idea that DNA from HRS cells is present in the serum of HL patients, and further suggest that serum EBV should be evaluated as a prognostic marker.

There is strong epidemiological evidence that an infectious agent may also be involved in the pathogenesis of young adult EBV-negative cHL cases from developed countries. It has been suggested that EBV may also cause these tumours by using a hit-and-run mechanism. Support for this idea comes from the

finding that most young adult patients, who are likely to have a good immune response to EBV, have EBV-negative HL. Analysis of EBV seroprevalence rates in an epidemiological study of young adult HL revealed that cases with EBV-negative HL were more likely to be EBV seronegative than controls. Additional studies clearly showed that some HL patients have never been infected by EBV. Quantitative PCR was used to look for the presence of deleted EBV genomes in a series of adult cases with both EBV-positive and negative HL. Subgenomic fragments were detected in equimolar proportions. This study found no evidence to support the idea that a hit-and-run mechanism involving EBV plays a role in the pathogenesis of HL.

In search of another infectious agent that may be involved in the transformation process of the young adult EBV-negative cases we looked for the presence of both herpesviruses and polyomaviruses using degenerate PCR strategies. Although sensitive, the assays were negative for novel viruses of these families. Future studies will therefore utilise other molecular techniques for virus discovery, as discussed in chapter 6, to search for a virus.

Serial Analysis of Gene Expression (SAGE) was used to investigate the gene expression profile in primary HRS cells from an EBV-positive and an EBV-negative case of cHL and also normal germinal centre (GC) B-cells. We looked for gene expression differences in the cHL libraries compared with the GC library to identify genes that may be involved in the disease pathogenesis. Protein Kinase C and Galectin 2 were two genes chosen for further investigation because of their plausible gene function and the apparent increased expression in the cHL cases. These genes may be involved in the disease pathogenesis; however, validation of the libraries is still ongoing and the results obtained require additional verification before any finite conclusions can be drawn. An additional aim of this study was to identify viral sequences that may be present in the EBV-negative case of cHL. We aim to increase the size of our libraries and also investigate SAGE tags generated that are not linked to known genes in the common databases. The use of primary

cHL material for global gene expression studies should help characterise the HRS cells and increase our understanding of the disease pathogenesis.

At present the aetiology of HL is still unknown and any infectious agent/s present in the EBV-negative cases remains elusive. Further studies using state-of-the-art molecular techniques for virus discovery are warranted.

Samenvatting

De klassieke variant van het Hodgkin lymfoom (cHL) is een vorm van kanker die wordt gekenmerkt door de aanwezigheid van een minderheid van tumor cellen, de zogenaamde Hodgkin en Reed-Sternberg (HRS) cellen, en een polymorfe cellulaire achtergrond van andere niet maligne infiltrerende cellen. Het lage percentage tumor cellen in het aangedane weefsel is gedurende lange tijd een belangrijke beperkende factor geweest voor de verdere karakterisering van deze cellen. Gedurende de laatste jaren is het duidelijk geworden dat de tumor cellen afkomstig zijn van pre-apoptotische kiemcentrum B cellen. De etiologie van het cHL is op dit moment nog niet bekend, we weten echter wel dat het Epstein-Barr virus (EBV) betrokken is bij de pathogenese in een deel van de gevallen. Het percentage EBV positieve gevallen varieert op basis van leeftijd, histologisch subtype en geografische locatie.

Op basis van de resultaten van een aantal studies is het duidelijk geworden dat DNA aanwezig in serum / plasma van patiënten vooral afkomstig is van de tumor cellen. Naar aanleiding van deze bevindingen hebben wij de hypothese geformuleerd dat de mogelijkheid om EBV DNA in serum van patiënten met EBV positieve cHL aan te kunnen tonen een belangrijk middel zou kunnen zijn voor klinische en epidemiologische studies. EBV DNA kon inderdaad aangetoond worden in 30 van de 33 patiënten met een EBV positief cHL en bij slechts 6 van de 26 patiënten met EBV negatief cHL ($p<0.001$). Sera van gezonde individuen waren altijd negatief en EBV DNA kon slechts bij 5 van de 12 patiënten met de ziekte van Pfeiffer worden aangetoond. Het aantal kopieën EBV dat kon worden aangetoond was variabel in de drie patiënten populaties. Op basis van een studie met een beperkt aantal patiënten bleek dat de aanwezigheid van EBV DNA in het serum correleerde met risicofactoren voor een slechte prognose. Deze resultaten bevestigen de hypothese dat DNA afkomstig van HRS cellen aanwezig is in het serum van cHL patiënten en suggereren dat aanvullende studies m.b.t. de

aanwezigheid van EBV DNA in het serum van patiënten als mogelijke prognostische marker zinvol zijn.

Er zijn duidelijke epidemiologische aanwijzingen dat ook bij EBV negatieve HL patiënten in de westerse landen een infectieuze factor betrokken is bij de pathogenese. Er is onderzoek gedaan of EBV ook in deze tumoren betrokken kan zijn geweest volgens het 'hit-and-run' mechanisme. Jong volwassenen die een goed werkend immuunsysteem hebben en waarschijnlijk ook een effectieve immuunrespons tegen EBV kunnen induceren, hebben vaak een EBV negatief HL. Serum analyse in het kader van een epidemiologische studie bij jong volwassenen toonde aan dat patiënten met een EBV negatief HL vaker negatief waren voor EBV dan gezonde controles. Aanvullende studies toonden bovendien aan dat sommige HL patiënten nog nooit geïnfecteerd waren geweest met EBV. Vergelijking van volwassen patiënten met EBV negatieve en positieve HL op basis van kwantitatieve PCR voor EBV DNA resulteerde in gelijke hoeveelheden in beide groepen. Op basis van deze studie kon geen bewijs worden gevonden voor het 'hit and run' mechanisme voor EBV in EBV negatieve HL patiënten. Het is dus aannemelijk dat EBV geen rol speelt bij de pathogenese van EBV negatieve HL.

Om een mogelijke andere infectieuze factor op te sporen die betrokken zou kunnen zijn bij de ontwikkeling van HL werd met behulp van gedegenereerde primers een PCR gedaan voor herpes virus en polyoma virus in EBV negatieve HL patiënten. Op basis van deze experimenten konden geen aanwijzingen gevonden worden voor de aanwezigheid van eventuele nieuwe varianten van deze virussen ondanks de hoge sensitiviteit van deze aanpak. Voor het aantonen van nieuwe of andere virussen in EBV negatieve HL zijn alternatieve screenings strategieën gewenst.

Gen expressie profielen van primaire HRS cellen van een EBV positieve en een EBV negatieve HL patiënt werden gemaakt met de 'Serial Analysis of Gene Expression' (SAGE) techniek. Ter controle werd ook een genexpressie profiel

gemaakt van normale kiemcentrum B cellen (CB). De expressie profielen van de HRS cellen werden vergeleken met het profiel van de CB om genen betrokken bij de pathogenese van HL te identificeren. Twee genen, Protein Kinase C en Galectin 2, werden geselecteerd voor verdere analyse vanwege hun functie en de verhoogde expressie in HRS cellen ten opzichte van CB. Deze genen zouden betrokken kunnen zijn bij de pathogenese van HL. De SAGE banken en de differentieel tot expressie komende genen zullen nog verder worden gevalideerd. Naast het identificeren van genen betrokken bij de pathogenese van HL, kunnen de expressie profielen ook worden gebruikt om eventueel aanwezige virale sequenties aan te tonen in de EBV negatieve HRS cellen, en op deze manier het mogelijk nu nog onbekende virus betrokken bij de pathogenese van HL op sporen. Hiervoor zullen in eerste instantie de SAGE banken van de HRS cellen en de CB nog verder worden uitgebreid. Door gen expressie profielen te maken van primair HL materiaal kunnen we de HRS cellen karakteriseren en zal onze kennis over de pathogenese van HL toenemen.

Op dit moment is de etiologie van cHL nog steeds niet opgehelderd en is met name nog onduidelijk of er wel of niet een andere infectieuze factor betrokken is bij de pathogenese van EBV negatieve cHL gevallen. Verdere studies met state-of-the-art moleculaire technieken zijn nodig voor het opsporen van andere en/of nieuwe virussen.

Abbreviations

BCR	B-cell receptor
BKV	BK virus
CB	centroblast
CCL17	TARC
cHL	classical Hodgkin lymphoma
EA	early antigen
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
GC	germinal centre
HCMV	human cytomegalovirus
HD	Hodgkin's disease
HHV	human herpes virus
HL	Hodgkin lymphoma
HLA	human leucocyte antigen
HRS	Hodgkin and Reed-Sternberg
HSV	herpes simplex virus
IAP	inhibitors of apoptosis
IFA	immunofluorescence assay
Ig	immunoglobulin
IL	interleukin
IM	infectious mononucleosis
JCV	JC virus
L&H	lymphocytic and histiocytic
LMP	latent membrane protein
MCHL	mixed cellularity Hodgkin lymphoma
NF-κB	nuclear factor kappa B
NLPHL	nodular lymphocyte predominance HL
NSHL	nodular sclerosis HL

PCR	polymerase chain reaction
qPCR	quantitative PCR
qRT	quantitative reverse transcriptase
RCA	rolling circle amplification
RDA	representational difference analysis
RTK	receptor tyrosine kinase
SAGE	serial analysis of gene expression
SOCS	suppressors of cytokine signalling
TGF-β	transforming growth factor-β
Th	T-helper
VCA	viral capsid antigen
VZV	varicella-zoster virus
XIAP	X-linked inhibitor of apoptosis

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Acknowledgements

I would like to thank my promotor Prof. dr. S. Poppema and copromotor Dr. J.H.M. van den Berg for giving me the wonderful opportunity to undertake my PhD with the University of Groningen. The warmth, encouragement and support shown to me in Holland will stay with me forever. I must particularly thank Joost Kluiver for all his help and expertise with the joint SAGE project and for his wonderful sense of humour. Everyone in the group made me very welcome and it felt like a home from home working in the Medical Center. A heart felt thank you to each and every one of you. Thank you in particular to my Paranimfen Marijke Niens and Çiğdem Atayar for their friendship and support. It is very much appreciated. Although mentioned as my copromotor I must also give a special thanks to Anke for her friendship, constructive criticism and also for the hospitality that she and Klaas have shown me. It was Anke who encouraged me to embark on this PhD and for that I am eternally grateful. Thankfully our on going collaborations and continued friendship will mean that the ties with Groningen remain strong.

I would like to thank my other promotor, Professor Ruth Jarrett for enabling me to undertake my PhD with the University of Groningen. Thank you for all your support, constructive criticism and continued friendship. I cannot express how much I appreciate the faith that you have shown in me over the years and how enjoyable that time has been. It has been a long and fruitful journey.

In addition, a heart felt thanks to all my colleagues in the LRF Virus Centre in Glasgow, for their encouragement and also for putting up with me for the duration of my PhD. Because of our closeness, I hope that my friends there know how much I value their opinions and treasure their friendship. It is a truly unique place to work.

A special thanks to Professor David Onions for your encouragement and kindness over the years. It is truly appreciated. Thank you also to my examiners Prof. dr. P.M. Kluin, Prof. dr. R. Küppers and Prof. dr. A. Rickinson for giving up their valuable time to read this thesis.

A big thank you also goes to my good friend Moyra Campbell for designing the book cover. Thank you to Heather O'Dowd for her help with the down sizing of figures for the book.

I would like to thank my Mum and family (Gahagan) and the McCallums for having faith and patience in me and for their never ending love. I wish that Dad was here to share the moment.

My final thanks go to my husband Paul, the love of my life, for his patience, encouragement and most importantly for giving me a wonderful family: His two beautiful girls Jennifer and Lindsay and our amazing son Stewart.